POST-TRANSLATIONAL CONTROL OF LUTEINIZING HORMONE SECRETION

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CONTENTS

For Isobel and Bill

DECLARATION

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Richard J. W. Currie

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ABSTRACT

The investigations detailed in this thesis were undertaken to determine the mechanisms regulating the post translational control of LH secretion in the ewe. LH is stored within dense core secretory granules in the gonadotrophs of the adenohypophysis. Studies on cellular ultrastructure and gene expression were conducted on pituitary glands obtained from Welsh mountain ewes at points throughout the oestrous cycle.

In order to determine if a structural priming response of gonadotrophin granules in preparation for the preovulatory LH surge was present in the sheep, fixed pituitary tissue was examined using immunogold labelling techniques under the electron microscope. In the luteal phase, 20% of gonadotrophs possessed granules located in one aspect of the cytoplasm juxtaposed to the nearest sinusoid, leading to the appearance of a polarised cell. The percentage of polarised cells increased throughout the cycle, reaching a peak of 90% in mid LH surge. Stereological analysis of granule diameter showed that exocytosis occurred in a size dependant manner. Initial secretion occurred from the 150-180nm size class with the larger granules released only during the preovulatory LH surge. LHβ mRNA abundance decreased from luteal through follicular phase, reaching its lowest value during the surge. The replenishment of intracellular LH stores occurred initially via the synthesis of the smaller granule size classes, first visible in the cytoplasm 48h after an induced LH surge. As cells refilled, displaying a morphology similar to a typical luteal phase gonadotroph at 96h post surge, the diameter of granules synthesized increased, approaching values observed in the late luteal phase. Throughout the refill phase, LHβ mRNA abundance did not change. These data suggest that the preovulatory LH surge, in the ewe, is not related to increased synthesis of LH but a progressive recruitment of gonadotrophs into a releasing state as indicated by the polarisation of secretory granules towards the abutting capillary. Furthermore, the absence of changes in steady state LHβ mRNA suggests that post transcriptional mechanisms operate to mediate the refilling of the LH stores.

The polarisation of gonadotrophs was prevented by the action of a GnRH antagonist. The actions of GnRH and oestradiol, independently, were able to induce polarisation. However, the direction of the granule translocation was incorrect in some 30% of cases indicating that *in vivo* the two hormone actions synergise to ensure the correct direction of the granule polarisation.

Chromogranin A, chromogranin B and secretogranin II, three members of the granin family of proteins, were colocalised within the same secretory granule as LH and in minimal amounts within granules of other pituitary cell types. Steady state mRNA levels of all three granins decreased in parallel with LHβ mRNA abundance approaching the LH surge. Post-surge, CgA and CgB mRNA levels remained constant. However, SgII mRNA abundance increased by 96h post surge suggesting an association with the larger granule classes present at that time. This association was confirmed by immunogold staining density analysis in which SgII appeared to be present at a higher concentration in the larger granule classes. The regulation of gene expression and the localisation of these proteins are suggestive of a role in the control of gonadotrophin granule size and structure.

LIST OF COMMONLY USED ABBREVIATIONS

ANOVA: analysis of variance

cAMP: cyclic adenosine monophosphate

B+(24, 48, 72, 96): 24h, 48h, 72h, 96h after the induction of an LH surge with buserelin

cDNA: complementary deoxyribonucleic acid

CgA: chromogranin A

CgB: chromogranin B

DAG: diacylglycerol

D_{max}: maximum profile/ granule diameter

E+(9, 24): 9h, 24h after behavioural oestrus

FSH: follicle-stimulating hormone

GnRH: gonadotrophin-releasing hormone

LH: luteinizing hormone

mRNA: messenger ribonucleic acid

PG/ PGF2α: prostaglandin F2α

PKC: protein kinase C

SgII: secretogranin II

Sn: secretoneurin

CHAPTER 1

LITERATURE REVIEW

Introduction

This literature review will describe the ovine oestrous cycle and the known control mechanisms of LH synthesis and secretion at both a pre- and post translational level. Following a general discussion on protein packaging and export, the Chapter will conclude by describing a class of proteins with a ubiquitous distribution throughout the neuroendocrine system including the adenohypophysis: the granins.

1.1. The Ovine Oestrous Cycle

The oestrous cycle is a rhythmic phenomenon during which a regular period of sexual receptivity, known as oestrus, occurs. In the Welsh mountain ewe, the oestrous cycle lasts for 17 days and occurs throughout the breeding season from October to March (Karsch 1980; Lincoln and Short 1980; Karsch and Moenter 1990). The cycle is initiated by the process of ovulation in which the oocyte is ejected from the preovulatory follicle as a consequence of a surge of luteinizing hormone (LH) secreted from the adenohypophysis. The hypothalamic hormone gonadotrophin-releasing hormone (GnRH) mediates the release of LH from the gonadotroph cells of the adenohypophysis (review: Thiery and Martin 1991).

The ovine oestrous cycle is composed of a luteal phase, which extends from day 2 to day 13 and a periovulatory phase from day 14 to day 1 (day 0 = oestrus; Baird and McNeilly 1981) containing the follicular phase from day 14 until the preovulatory gonadotrophin surge.

1.1.1. The Luteal Phase

High concentrations of progesterone produced from the corpus luteum characterise the luteal phase. Following ovulation, the granulosa and thecal cells of the recently ruptured follicle undergo luteinization and begin to secrete progesterone (Hauger *et al* 1977; Hansel and Convey 1983; Rowlands and Weir 1984). The maximum concentration

of progesterone secreted is 3-4ng/ml which is present in the circulation between day 6 and 12 of the cycle (Hauger *et al* 1977; Karsch *et al* 1980b). Progesterone, together with oestradiol negatively regulates LH pulses at this stage of the cycle (see section **1.3.1.2.**).

At this time, waves of developing follicles are present in the ovary (McNeilly *et al* 1992). The levels of follicle-stimulating hormone (FSH) during luteal phase (Pant *et al* 1977) are sufficient to allow follicle growth up to 4mm in diameter before regression (England *et al* 1981). FSH is able to stimulate follicle growth in the absence of LH pulses (Picton *et al* 1990). Waves of follicular growth occur every 4-6 days during the luteal phase (Smeaton and Robertson 1971; Brand and De Jong 1973; Bister and Paquay 1983) and it has been proposed that these periods of follicular growth are not completed due to inadequate gonadotrophin support (Salamonsen *et al* 1973; Findlay and Clarke 1987). It is clear that progesterone controls the length of the luteal phase since immunization against progesterone induces ovulation around 4 days later (Thomas *et al* 1987). Furthermore, towards the end of the luteal phase, progesterone prevents the high oestradiol levels present from triggering an LH surge and also arrests follicle maturation (Baird and McNeilly 1981; Glencross 1987).

In the ewe, luteolysis occurs at around day 12 of the cycle and is initiated by prostaglandin F2α (PGF2α) (Baird 1978; Baird and McNeilly 1981). The luteolysin is released from the endometrium (Flint and Hillier 1975) and may be regulated by oxytocin (Roberts *et al* 1976; Sheldrick and Flint 1985; Hooper *et al* 1987). The luteolytic effects of PGF2α may be augmented by oestradiol as oestradiol administration induces PGF2α secretion from the uterus (Hixon *et al* 1983) whilst ablation of oestrogenic follicles by X-irradiation results in a lengthened luteal phase (Karsch *et al* 1970).

1.1.2. The Follicular Phase

Subsequent to luteolysis, the resulting precipitous decline in progesterone concentration occurs concurrently with a wave of follicle growth (Smeaton and Robertson 1971). One of this cohort of follicles will proceed through maturation and ovulate (Herriman *et al* 1979). LH secretion is stimulated at this stage of the cycle due to the removal of the negative influences of progesterone and the increased concentrations of oestradiol from the developing follicles (Webb and England 1982; Karsch *et al* 1983). At the same time, FSH concentrations decrease due to the negative effects of oestradiol (Baird and McNeilly 1981; Fraser *et al* 1981; McNeilly 1984; Wallace *et al* 1988; Campbell *et al*

1990) and increasing inhibin production from the developing follicles (Baird *et al* 1991). Studies on FSHβ gene expression (Gharib *et al* 1990; Mercer 1990) suggest that the rate of FSH secretion (review: Price 1991) is closely related to its synthesis (McNeilly 1988; Brooks *et al* 1992) and gene expression (Phillips *et al* 1988). This decline in FSH concentrations may be involved in the selection of the dominant follicle which proceeds to ovulation (Scaramuzzi *et al* 1993).

The increasing LH pulse frequency throughout the follicular phase (see section **1.3.1.**) increases ovarian oestradiol production (Baird 1978) via the heightened secretion of androgens from the thecal cells which are converted by the aromatase enzyme in the granulosa cells to oestradiol (Baird 1977; McNatty 1981). The elevated oestradiol concentration induces behavioural oestrus and the preovulatory LH surge towards the end of the follicular phase (Hauger *et al* 1977; Pant *et al* 1977) with ovulation occurring some 24h later (Cumming *et al* 1971).

1.1.3. The Periovulatory Period

A surge type release of both LH and FSH occurs around the time of behavioural oestrus (Baird and McNeilly 1981). The generation of the preovulatory LH surge is mediated by increasing concentrations of oestradiol which cause an increased GnRH pulse frequency and surge (Clarke *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994) and a sensitisation of the pituitary gland to GnRH (Clarke and Cummins 1984). In the approach to the LH surge in the rat, increased numbers of maturing gonadotrophs express GnRH receptors as indicated by the presence of avidin-peroxidase detected biotinylated GnRH colocalised with LH immunoreactivity (Childs *et al* 1994). The mutual positive feedback loops of LH and oestradiol increase their own plasma concentrations, the effect culminating in the LH surge (see section **1.3.1.**).

During the surge, plasma LH concentrations of greater than 5ng/ml degrade the follicular aromatase (Moor 1974; Baird and McNeilly 1981) leading to a decrease in oestradiol production. In tandem with this, a decrease in follicular LH receptor number (Webb and England 1979, 1982), which occurs at this time, leads to an increase in testosterone and androstenedione concentrations (Baird *et al* 1981).

Following the initial gonadotrophin surge, a second rise of FSH occurs around 20-30h later, known as the FSH rebound (Salamonsen *et al* 1973; Baird and McNeilly 1981). The mRNAs for LHβ, FSHβ and α subunit increase during an induced FSH rebound

(Brooks *et al* 1992) suggesting that the second FSH surge is due to *de novo* synthesis of FSH. The FSH surge may function to recruit the first developing wave of follicles in the next luteal phase (Smeaton and Robertson 1971; Pant *et al* 1977; McNatty *et al* 1981; Lahlou-Kassi *et al* 1984).

1.2. Hypothalamic Control of LH Secretion

1.2.1. The Synthesis and Release of GnRH

The decapeptide GnRH is a product of the post-translational cleavage of a larger precursor molecule. The precursor is composed of a signal sequence, the ten amino acid-long GnRH sequence, a post-translational processing site and a peptide 56 amino acids in length known as gonadotrophin-releasing hormone-associated peptide (GAP) (Seeburg and Adelman 1984). The structure of GnRH is identical in mammalian species, with GAP approximately 85% conserved between human, rat and mouse (Seeburg *et al* 1987). Low concentrations of GAP stimulate prolactin release from rat pituitary cells whilst higher doses lead to an inhibition (Seeburg *et al* 1987). Although GAP is able to stimulate LH secretion, it does so with a lower potency than GnRH (Nickolics *et al* 1985).

GnRH is localised in populations of neurones in the mediobasal hypothalamus, anterior hypothalamic and preoptic areas, the septal complex and other regions of the fore brain (Sherwood *et al* 1976; ; Jennes and Stumpf 1980; Silverman *et al* 1982; Witkin *et al* 1982; Lehman *et al* 1986; Silverman 1988). There are around 2500 such GnRH neurones in the sheep brain (Caldani *et al* 1988). The decapeptide is transported along the axons to its storage site in the nerve terminals of the median eminence. GnRH is secreted into the hypophyseal portal blood stream which transports the hormone to the adenohypophysis where it stimulates the secretion of the gonadotrophins LH and follicle stimulating hormone (FSH) (Dierschke *et al* 1970; Schally *et al* 1971; Aiyer *et al* 1974a; Clarke *et al* 1978; Lincoln and Fraser 1979; Fraser and McNeilly 1982).

The release of GnRH from the median eminence is regulated by the 'GnRH pulse generator' which has been localised in the region of the arcuate nucleus of the mediobasal hypothalamus (Goodman and Karsch 1981; Knobil 1981). Inhibition of GnRH release has been demonstrated by prolactin addition to GnRH-containing neuronal cell lines (Milenkovic *et al* 1994) and administration of a met-enkephalin

analogue to women (del-Pozo and Marin-Perez 1985). Opioid antagonists increase LH pulse frequency during the luteal phase and LH pulse amplitude during the follicular phase in the ewe (Brooks *et al* 1986; Currie and Rawlings 1987; Whisnant and Goodman 1988) by acting at the hypothalamic level (Whisnant *et al* 1991) to increase GnRH secretion (Horton *et al* 1987; Caraty *et al* 1987). The GnRH-containing neurones are able to secrete in a pulsatile fashion *in vitro* which suggests that an intrinsic rhythmicity may be co-ordinated by a separate rhythm-generating unit (Martin 1984). The pulse generator stimulates the secretion of GnRH in a pulsatile manner. Early evidence to support this mode of release came from the measurement of GnRH in pituitary stalk portal blood samples (Carmel *et al* 1976). Similar measurements have been made in the rat (Sarkar and Fink 1980; Soper and Weick 1980). The use of GnRH antibodies to immunoneutralize GnRH abolishes pulsatile LH secretion in the ewe (Clarke *et al* 1978; McNeilly *et al* 1984), ram (Lincoln and Fraser 1979), ovariectomized female rats (Snabels and Kelch 1979), castrated male rats (Ellis *et al* 1983) and stumptailed macaques (Fraser *et al* 1982; Fraser 1986). In the female rat and sheep, and gilts, this immunoneutralization also inhibits the naturally occurring or steroid induced preovulatory LH surge (Koch *et al* 1973; Arimura *et al* 1974; Blake and Kelch 1981; Fraser and McNeilly 1982, 1983; Esbenshade and Britt 1985). The "hypothalamic clamp" studies performed by Knobil (1980), in which radiofrequency lesions were placed in the medial basal hypothalamus of ovariectomized rhesus monkeys, demonstrated that restoration of the GnRH input via exogenous pulsatile administration re-established normal plasma gonadotrophin secretory profiles and regular menstrual cycles. The studies also demonstrated that the nature of the GnRH input was important in the regulation of gonadotrophin secretion: continuous GnRH administration produced an initial increase in gonadotrophin secretion followed by a refractory period during which the pituitary appeared unresponsive to GnRH. Plasma LH and FSH concentrations declined (Belchetz *et al* 1978). This effect was reversed upon the readministration of GnRH pulses. These findings have also been reported in ovariectomized, hypothalamo-disconnected ewes (Clarke *et al* 1984) and clinical conditions of GnRH deficiency in man (Crowley and McArthur 1980; Leyendecker *et al* 1980; Shoemaker *et al* 1981; Santoro *et al* 1986).

Sampling of the conscious animal and the measurement of GnRH pulses has been demonstrated in sheep using the portal access model (Clarke and Cummins 1982), push-pull perfusion of the median eminence (Levine and Ramirez 1980) or the third ventricle (Van Vugt *et al* 1985) and pituitary effluent sampling in the horse (Alexander and Irvine 1987). Perhaps the best method to date for the ewe was detailed by Moenter *et al*

(1990). A modification of a previous method (Caraty and Locatelli 1988), it apparently leads to a lesser degree of damage to the surrounding neural tissue since normal LH pulse amplitude and preovulatory surges have been measured peripherally, following surgery. This model has shown GnRH pulse frequencies of 0.25±0.05 pulses/h in luteal phase with a mean amplitude of 3.2±1.0 pg/min (Moenter *et al* 1991). These authors reported that, in the early follicular phase, the GnRH pulse frequency exceeded 1pulse/h at similar amplitudes to those observed in luteal phase. Within the follicular phase pulse frequency increased and pulse amplitude decreased during two 6h periods 24-28h and 8-2h before the LH surge. The existence of a GnRH surge, which accompanied the LH surge, was reported by Clarke *et al* (1987). However, these authors only observed a GnRH surge in three out of six animals investigated in the follicular phase. This may have been due to the lack of sufficient blood samples to determine a presurge baseline concentration (I.J. Clarke, personal communication - cited in Moenter *et al* 1991). A large surge release of GnRH, reaching a plasma concentration of around 20pg/ml, was observed in 11 out of the 12 ewes studied by Moenter *et al* (1991). The duration of the GnRH surge extended beyond the termination of the LH surge.

A one-to-one relationship between large GnRH and LH pulses has been demonstrated (Clarke and Cummins 1982; Levine *et al* 1982; Van Vugt *et al* 1983). The same studies reported GnRH pulses that were not associated with LH pulses, a phenomenon that has recently been described approaching the onset of the GnRH surge as GnRH pulse frequency increases (Moenter *et al* 1991).

1.2.2. GnRH Action in the Pituitary

GnRH causes a cellular response via binding to a specific, high affinity receptor localised on the plasma membrane of the gonadotroph cells which leads to the activation of intracellular second messenger systems (Marian and Conn 1983; Clayton 1987; Conn *et al* 1987a). The GnRH receptor is a member of the seven transmembrane receptor class. In the mouse, the receptor is a 327 amino acid protein with a molecular weight of 37,683 and an extremely truncated cytoplasmic C terminus (Tsutsumi *et al* 1992; Reinhart *et al* 1992). A similar truncated C terminal structure is present in the rat (Eidne *et al* 1992; Perrin *et al* 1993) and the sheep, human and cow (Kakar *et al* 1992, 1993a; Brooks *et al* 1993; Illing *et al* 1993; Chi *et al* 1993).

Activation of the receptor leads to mobilisation of extra- and intracellular calcium (Clapper and Conn 1985; Leong *et al* 1986; Chang *et al* 1986; McArdle *et al* 1992).

Elevation of intracellular calcium concentrations is biphasic in nature with an initial peak being followed by a prolonged plateau phase (Turgeon and Waring 1986; Anderson *et al* 1992, 1993). Mobilisation of calcium from intracellular stores produces the initial rapid calcium transient whilst extracellular calcium enters via a specific plasma membrane channel (Hopkins and Walker 1978; Marian and Conn 1979; Barbarino and DeMarinis 1980; Veldhuis *et al* 1985; Conn *et al* 1987a) and contributes predominantly to the plateau phase. The calcium oscillations may be central to LH secretion as calcium ionophores, ionomycin, depolarising agents, calcium channel activators and calcium-loaded liposomes stimulate LH secretion with a similar efficacy to GnRH (Conn *et al* 1979, 1980a,b, 1987b; Conn and Rogers 1980; Conn 1983). It has recently been suggested that activin, which stimulates the release of FSH, may suppress the activity of GnRH receptor-associated calcium channels (Katayama and Conn 1994).

GnRH receptor activation also initiates an increase in phospholipid metabolism (Snyder and Bleasdale 1982; Raymond *et al* 1984) via guanosine triphosphate proteins (G proteins) (Cantau *et al* 1980; Andrews *et al* 1986; Perrin *et al* 1989; Hawes and Conn 1992a) leading to breakdown of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Noar *et al* 1986; Morgan *et al* 1987; Huckle and Conn 1988; Keisel 1993). Initial studies suggested that production of IP_3 may be a requirement for generation of the initial intracellular calcium spike as its application induced the release of calcium from cytosolic organelles within the pituitary (Guillemette *et al* 1987). Subsequent studies have determined that calcium mobilisation occurs from the endoplasmic reticulum whilst DAG activates protein kinase C (PKC) thus stimulating an extracellular calcium influx (Shangold *et al* 1988; Stojilkovic *et al* 1988; Andrews *et al* 1990; Izumi *et al* 1990; Dan-Cohen *et al* 1990). Although prolonged LH secretion is known to require the presence of extracellular calcium (Bates and Conn 1984), the initial secretory phase of LH release is predominantly mediated via intracellular stores (Chang *et al* 1988) as the depletion of intracellular calcium abolishes this early release (Hansen *et al* 1987). It is not clear if IP_3 functions as a classical second messenger in the transduction of the GnRH secretory signal. Although GnRH acts to mediate IP_3 turnover (Snyder and Bleasdale 1982; Andrews and Conn 1986; Huckle and Conn 1988) it has been demonstrated that GnRH and sodium fluoride-mediated LH release may be uncoupled from IP_3 turnover (Hawes *et al* 1992). Furthermore, inositol phosphate accumulation and the desensitization of the gonadotroph can also be uncoupled (Hawes and Conn 1992b). Thus elevated intracellular IP_3 concentrations are associated with, but not essential for, LH release.

The production of DAG sensitizes the enzyme protein kinase C (PKC) to activation by calcium (Kishimoto *et al* 1980). Activation of PKC leads to LH release, an effect observed experimentally with the use of phorbol esters (Smith and Vale 1980; Smith and Conn 1984). This LH release mechanism does not require extracellular calcium (Conn *et al* 1985; Naor and Eli 1985) or calmodulin and is elevated by calcium ionophores presumably through an increased activation of PKC (Harris *et al* 1985). In the physiological context, GnRH receptor occupation activates PKC and redistributes it from the cytosolic to the plasma membrane (Kraft and Anderson 1983; Andrews and Conn 1986; Naor *et al* 1986; Hirota *et al* 1985; Naor *et al* 1985; McArdle and Conn 1986). As with IP_3, PKC contributes to but is not essential for GnRH-mediated LH release. Using cell culture systems, pretreatment with phorbol esters to decrease PKC activity does not affect LH release caused by calcium mobilizing stimuli (McArdle *et al* 1987; McArdle and Conn 1988). Also, phorbol esters stimulate LH release to a lesser degree than GnRH (Conn *et al* 1985; Harris *et al* 1985).

Recently a novel peptide known as pituitary adenylate cyclase activating polypeptide (PACAP) has been shown to increase concentrations of cAMP, inositol phosphate and intracellular calcium in α T3-1 cells, synergizing with low concentrations of GnRH (Schomerus *et al* 1994). However, *in vivo* studies in men have shown PACAP to have no effect on GnRH-stimulated LH release (Hammond *et al* 1993).

The effects of GnRH on the gonadotroph are also influenced by the abundance of GnRH receptors. The receptor will down-regulate in response to continuous stimulation with high concentrations of its endogenous ligand (Conn *et al* 1983; Zilberstein *et al* 1983) possibly by internalizing the occupied receptors (Braden *et al* 1989). In contrast, low concentrations of GnRH stimulate the synthesis and expression of increased numbers of its own receptor (Conn *et al* 1983; Loumaye and Catt 1983; Braden and Conn 1990) as does progesterone (Emons *et al* 1992). *In vitro* data from rat cultured pituitary cells indicates that pulsatile GnRH input is required to increase GnRH receptor mRNA levels (Kaiser *et al* 1993). Ovine cultured pituitary cells show an increase in GnRH receptor numbers in response to oestradiol and inhibin which is attenuated with progesterone (Sealfon *et al* 1990; Laws *et al* 1990). Upregulation may involve PKC (Naor *et al* 1987; Huckle *et al* 1988; Braden *et al* 1991) but not extracellular calcium (Braden and Conn 1990). Small amplitude, rapid pulses of GnRH immediately prior to the preovulatory LH surge (Moenter *et al* 1991) may therefore serve to sensitize the gonadotrophs to the GnRH surge that accompanies the preovulatory LH surge (Clarke *et al* 1987; Moenter *et*

al 1991). GnRH receptor mRNA increases at this time during the oestrous cycle (Brooks *et al* 1993, Wu *et al* 1994), leading to an increase in GnRH receptor numbers (Savoy-Moore *et al* 1980, 1981; Moss *et al* 1981; Adams *et al* 1981; Crowder and Nett 1984). A similar increase in GnRH receptor mRNA occurs in the rat immediately prior to the LH surge (Bauer-Dantoin *et al* 1993).

Due to the large numbers of GnRH receptors in the pituitary, it may be that regulation of post receptor transduction mechanisms is of greater importance than modulation of receptor numbers in the control of gonadotroph sensitivity to GnRH (Clayton 1987).

1.3. Luteinizing Hormone Secretion

Luteinizing hormone is stored within electron-dense secretory granules in the cytoplasm of gonadotroph cells located in the adenohypophysis. During the oestrous cycle, LH is secreted in distinct pulses which directly reflect the GnRH secretory signal from the hypothalamus (Clarke and Cummins 1982). In the sheep, the frequency of pulses exhibits great variation depending upon the season. In anoestrus, LH is released at a rate of 1 pulse/24h (Yuthasastrakosol *et al* 1977), increasing to more than 1 pulse/h during the late follicular phase (Baird 1978). In Suffolk ewes, the pulsatile characteristics of LH secretion were lost in the period immediately prior to the preovulatory LH surge (Moenter *et al* 1991) whilst in Finn/Dorset cross ewes, pulsatile LH release remained present until the surge (Wallace *et al* 1988). An LH pulse contains approximately 2μg of LH in Finnish Landrace/Merino cross ewes (McNeilly *et al* 1982), corresponding to around 0.3% of pituitary LH content (Fraser *et al* 1981).

A biphasic release of LH in response to GnRH infusion has been reported in hamsters (Arimura *et al* 1972), humans (Bremner and Paulsen 1974; Hoff *et al* 1977), sheep (Kanchev *et al* 1984; Bremner *et al* 1976; Bremner *et al* 1980) and rats (Edwardson and Gilbert 1975; DeKoning *et al* 1976). It has been proposed that this corresponds to an initial secretory phase in which LH stored in releasable form is secreted, the releasable pool, whilst the second phase constitutes both LH synthesised *de novo* and that has undergone post-translational processing (Yen 1977; Liu and Jackson 1978). Since the LH pulse amplitude varied inversely with LH pulse frequency (Baird 1978; Karsch *et al* 1983; Clarke *et al* 1984; Clarke and Cummins 1985) it has been proposed that the magnitude of the LH response to each GnRH pulse is therefore an index of the size of the releasable pool (Clarke and Cummins 1985).

In some species, the releasable pool may be a reflection of the localisation of the LH immunopositive secretory granules within the cytoplasm. In cultured rat pituitary cells, brief treatment with GnRH for 15-30mins induces the migration of secretory granules to the periphery of the gonadotroph near the plasma membrane with a decrease in the electron density of the granule matrix (Tixier-Vidal *et al* 1975). Studies on mouse hemipituitaries, which were exposed to GnRH for two hours, demonstrated that the peripheralisation of secretory granules was associated with the GnRH priming effect on LH release (Lewis *et al* 1986) and suggests that, in this species, the releasable pool may be the number of gonadotrophs which have peripheralised secretory granules before the GnRH bolus is administered. Under *in vitro* conditions, the translocation of the granules is reversed following GnRH withdrawal (Morris *et al* 1986). In the mouse gonadotroph, exocytosis of secretory granules occurs predominantly from the area of the cell membrane directly adjoining the vascular system (Durnin and Morris 1992c).

The priming effect, and hence granule movement, may be mediated by cytoskeletal microfilaments since disruption of microfilaments, but not microtubules, reduces significantly GnRH-induced LH release (Adams and Nett 1979). During the priming process, the microfilaments elongate and undergo a change in orientation such that the incident angle between the filament and the plasma membrane is reduced (Lewis *et al* 1985). The significance of the altered microfilament orientation with respect to granule exocytosis is unclear. Microfilaments running parallel with the cell membrane would appear to act as a barrier, impeding the transport of granules towards exocytotic sites.

1.3.1. Control of LH secretion

The major regulatory system which controls LH secretion involves classical feedback loops. The existence of long, short and ultrashort feedback loops have been proposed (Clarke 1987). The long loop feedback refers to the regulatory actions of oestradiol and progesterone on the hypothalamus and the adenohypophysis whilst the short and ultrashort loops describe the mechanisms by which pituitary and hypothalamic hormones self-regulate their own secretion (Karsch 1984).

1.3.1.1. Oestradiol

Oestradiol exerts both negative and positive regulatory effects on the secretion of LH. Negative feedback is observed at the level of the hypothalamus and the pituitary gland.

At the pituitary gland, oestradiol negative feedback was first observed when infusion of oestradiol was shown to suppress LH pulses in ovariectomised rhesus monkeys (Yamaji *et al* 1972). The suppression may be sustained for many months if oestradiol is maintained (Karsch *et al* 1973a, b). The removal of the effects of circulating oestradiol via ovariectomy leads to increased LH pulse frequency in monkeys and humans (Dierschke *et al* 1970; Knobil 1974, 1980), sheep (Butler *et al* 1971, 1972; Reeves *et al* 1972; Caraty and Locatelli 1988) and rats (Gay and Sheth 1972). In the rat and the sheep, this increased pulse frequency together with increased LH pulse amplitude results in increased circulating concentrations of LH over several weeks (Niswender *et al* 1968; Gay and Midgley 1969; Reeves *et al* 1972).

It is of note that, in the ewe, the negative effects of oestradiol differ according to the reproductive status of the animal. During anoestrus, oestradiol reduces LH pulse frequency, a hypothalamic effect, whilst during the oestrous cycle, the primary effect appears to be directed towards the reduction of LH pulse amplitude, a direct effect at the pituitary (Legan *et al* 1977; Goodman *et al* 1981a; Webster and Haresign 1983; Robinson *et al* 1985). Oestradiol in suppressing LH pulse amplitude (Goodman and Karsch 1980; Rawlings *et al* 1984) may operate by altering the size of the releasable pool since treatment of ovariectomized ewes (Goodman and Karsch 1980) and rats (Libertum *et al* 1974) with oestradiol decreases gonadotroph responsiveness to GnRH.

Microinjections of oestradiol directly into the hypothalamus block pulsatile LH secretion (Ferin *et al* 1974) whilst neurones which accumulate oestradiol have been located in the medial basal hypothalamus, in the vicinity of GnRH-secreting neurones in the rhesus monkey (Pfaff *et al* 1976; Silverman *et al* 1982). Subsequently, oestradiol was shown to act by stimulating a population of catecholaminergic neurones which inhibit GnRH secretion (Meyer and Goodman 1986). Systemic LH levels and LH pulse frequency rose in response to norepinephrine and dopamine receptor antagonists in anoestrus ewes (Meyer and Goodman 1985). Similar results have been obtained in ovariectomized and ovariectomized, oestradiol-treated ewes (Meyer and Goodman 1986; Kao *et al* 1992; LeCorre and Chemineau 1993). As these drugs were ineffectual during the breeding season, it is probable that catacholaminergic inhibitory systems only operate during anoestrus (Meyer and Goodman 1985). It is likely that the catecholamine action originates from dopaminergic neurones with axons that synapse directly onto GnRH nerve terminals in the median eminence in the sheep (Kuljis and Advis 1989). The perikarya of these neurones may reside in two fairly distinct regions just dorsal to the

caudal aspect of the optic chiasma and in the retrochiasmatic region medial to the optic tracts (Havern *et al* 1994).

The positive feedback effects of oestradiol are central to the generation of the preovulatory LH surge as immunization against oestradiol in the ewe prevents ovulation (Rawlings *et al* 1978, 1979). In addition, an LH surge is induced following bolus parenteral oestradiol administration to anoestrus ewes (Goding *et al* 1969; Bolt *et al* 1971) and ovariectomized ewes (Scaramuzzi *et al* 1971). A similar response has been reported in lactating females but the magnitude of the LH response was significantly reduced (Smith 1978; Coppings and McCann 1979; Wright *et al* 1980).

The LH surge is composed of high amplitude, high frequency LH pulses (Gallo 1981; Rahe *et al* 1980; Karsch *et al* 1983; Djahanbakhch *et al* 1984) at a frequency of around 1pulse/15mins. Theoretical considerations have suggested that an increase in LH pulse frequency is required for the initiation of the surge (Martin *et al* 1987). In this context, oestradiol may act to increase synthesis of the gonadotrophins and GnRH receptor numbers (Karsch 1987) thus allowing the pituitary to transduce the high frequency GnRH input present at this time (Moenter *et al* 1991). In contrast, it has also been proposed that the oestradiol augments the duration of each LH secretory event near the surge and hence increases the total mass of secreted hormone (Quyyumi *et al* 1993). The transfer from the negative to the positive modulatory effects of oestradiol may be a threshold phenomenon. It has been shown that, in women and monkeys, follicular phase plasma concentrations of oestradiol above 200pg/ml for 48h produce an induced LH surge (Yen and Tsai 1971; Monroe *et al* 1972; Yamaji *et al* 1972; Karsch *et al* 1973c).

1.3.1.2. Progesterone

Progesterone does not modulate pituitary sensitivity to GnRH directly (Goodman and Karsch 1980) but does decrease GnRH pulse frequency at the hypothalamic level (Cumming *et al* 1972). In the luteal phase, when progesterone levels are high, the steroid decreases LH pulse frequency (Goodman and Karsch 1980; Goodman *et al* 1981a; Tamanini *et al* 1986). This process requires oestrogen priming since LH pulse frequency is not suppressed in the late luteal phase of ewes treated with bovine follicular fluid, to suppress FSH secretion and hence follicular oestradiol production even though progesterone was maintained (Wallace *et al* 1985, 1988; Wallace and McNeilly 1986). The oestrogen priming results in sensitisation of the GnRH pulse generator to the progesterone signal (Karsch 1984).

Investigators have recently identified nitric oxide as an inhibitor of GnRH-induced LH release from gonadotrophs, the nitric oxide synthase enzyme and mRNA increasing after gonadectomy (Ceccatelli *et al* 1993). The authors speculated that this substance may participate in producing the pulsatile secretion patterns of the gonadotrophins. A different study indicates that the ability of norepinephrine to induce GnRH release is mediated by α1-adrenergic activation of nitric oxide synthase (Rettori *et al* 1993). The resultant nitric oxide produced induced prostaglandin E_2 release which facilitated exocytosis of GnRH from terminals in the median eminence to effect LH release from the pituitary (Rettori *et al* 1993).

1.4. Gonadotrophin Synthesis

1.4.1 Generalised Mechanisms of Protein Packaging and Export

Following the interaction of mRNA species with the ribosomes on the rough endoplasmic reticulum (RER) membrane, the nascent peptide is synthesized by the elongation of a series of amino acids presented by transfer RNA in a set order corresponding to that indicated by the triplet codons of nucleotides which compose the mRNA molecule. Proteins for export in mammalian cells enter the lumen of the RER via a translocation process across the RER membrane, through a proposed translocation channel, the translocon (Walter and Lingappa 1986; Simon and Blobel 1991; Crowley *et al* 1993), which is mediated via a signal targeting sequence on the nascent peptide (Walter and Lingappa 1986; Randall *et al* 1987). The targeting sequence is recognised and bound by the signal recognition particle (SRP) prior to the association of the SRP/nascent peptide complex with a receptor, known as the docking protein (DP), on the RER membrane (Walter and Lingappa 1986; High and Dobberstein 1992). The SRP is composed of a 7S RNA and six proteins of molecular masses 9K, 14K, 19K, 54K, 68K and 78K respectively (Walter and Lingappa 1986). The 9K and 14K subunits are able to arrest the elongation of the nascent peptide on the ribosome, whilst the 68K-72K complex facilitates the binding of the SRP to the RER membrane (Seigel and Walter 1988). The 54K subunit binds to the peptide targeting sequence (Kreig *et al* 1986; Wiedmann *et al* 1987) and may control insertion into the translocon (Miller *et al* 1993). The DP is composed of a and b subunits of 72K and 30K respectively (Tajima *et al* 1986) with the association of the SRP complex to the DP requiring GTP (Bernstein *et al* 1989; Connolly and Gilmore 1989; Romisch *et al* 1989).

Following passage through the translocon, and entry into the RER lumen, nascent peptide is modified by two enzymes known as the signal peptidase complex and the oligosaccharyl-transferase complex, which may be structural components of the translocon (Evans *et al* 1986; Kelleher *et al* 1992). In the RER lumen, chaperone molecules associate with secretory peptides, stabilising unfolded or partially folded tertiary structures within each peptide. An example of such a molecule is immunoglobulin heavy chain binding protein (BiP) (Pelham 1986). BiP and other resident RER lumenal proteins possess a series of C terminal residues: Lys-Asp-Glu-Leu known as KDEL (Pelham 1989). As the secretory product passes through the endomembrane system to the trans Golgi network (TGN) , the KDEL motif is used to target the lumenal chaperones back to the RER (Munro and Pelham 1987; Jackson *et al* 1993). Recently, the ERGIC (p) 53 protein, a marker for the endoplasmic reticulum Golgi intermediate compartment (ERGIC), was found to have a KDEL type motif (Schindler *et al* 1993). The reason for its specific localisation within the ERGIC is therefore not clear. The exact function of the ERGIC compartment is not known but it may play a role in protein folding (Hauri 1992). Transport from the RER to the TGN is GTP dependent. A ras-like GTP-binding protein, Sar1p, which is required for RER-TGN transport associates with the RER in the presence of Sec12, a GDP/GTP exchange protein (Barlow and Schekman 1993).

In the TGN, chaperone proteins return to the RER by means of the KDEL motif. Proteins shuttle through the TGN by budding from one Golgi stack to another (Rothman and Orci 1992). This process is thought to involve a protein class known as the coatomers (Orci *et al* 1993). Structurally, these proteins are similar to β adaptin, a component of the vesicular clathrin coat (Duden *et al* 1991). It has been suggested that the energy required to assemble the coatomer complexes on membranes may lead to deformation and hence budding (Takizawa and Malhotra 1993). Many GTP binding proteins may also be required for the budding process including rab1a, rab1b and rab 2 (Balch *et al* 1992) and ADP-ribosylating factor (Palmer *et al* 1993).

Following the exit from the TGN, the vesicle travels through the cytoplasm to the plasma membrane. To effect exocytosis, the secretory vesicle must fuse with the plasma membrane. The membranes are thought to be brought into close apposition by a fusion scaffold (Fernandez *et al* 1992). The fusion pore (Almers and Tse 1990) allows the secretion of the granule contents and the net movement of plasma membrane into the granule membrane (Monck *et al* 1990).

Following exocytosis, vesicular membrane targeting proteins and RER proteins from the TGN are retrieved and recycled (Sandvig *et al* 1992; Sollner *et al* 1993).

1.4.2. Gonadotrophin Structure

Both LH and FSH are members of a family of glycoprotein hormones which possess common structural properties. Together with thyroid stimulating hormone (TSH) and chorionic gonadotrophin (CG), they are heterodimers composed of a common α subunit and a β subunit which confers biological specificity (Papkoff and Samy 1967; de-la-Llosa and Justisz 1969; Pierce and Parsons 1981).

The gonadotrophin subunits are encoded by separate genes which are localised on different chromosomes (Chin 1986). Following translation, the α and β subunits combine in the lumen of the rough endoplasmic reticulum where the oligosaccharides, which have been co-translationally attached to asparagine residues on each of the subunits, are initially processed (Hoshina and Boime 1982). The glycosylated heterodimer passes through the endomembrane system, probably via the endoplasmic reticulum Golgi intermediate compartment (ERGIC) (Chavrier *et al* 1990; Schweizer *et al* 1991) to the trans Golgi network where post-translational modification of the carbohydrate structure occurs (Parsons *et al* 1983). It has been demonstrated that a signal motif present on the α subunit is recognised by the pituitary specific enzyme Gal-N-acetyl transferase. Interaction of the motif with the enzyme is mediated by the associated β subunit thus facilitating different degrees of glycosylation between LH and FSH (Smith and Baenziger 1988). Expression of the transferase enzyme is increased in the presence of oestradiol (Dharmesh and Baenziger 1993). A greater degree of glycosylation increases the ability of the hormone to produce cAMP in the target cell (Sairam and Bhargavi 1985) which may be related to an increase in circulatory half life (Morell *et al* 1971). Post translational processing of gonadotrophins therefore produces differing isoforms of the hormones (Wilde 1987; Ulloa-Aguire *et al* 1988; Stumpf *et al* 1992a), the more basic forms of which are preferentially secreted in response to GnRH (Reader *et al* 1983; Wide and Hobson 1983; Chappel *et al* 1983) leading to increased bioactivity *in vitro*. In contrast, other investigators reported a reduced percentage of the basic LH isoforms were secreted in response to GnRH (Zalesky and Grotjan 1991) with immunization against GnRH leading to an increase in the most acidic LH isoform in the pituitary (Zalesky *et al* 1993).

1.4.3. Regulation of Gonadotrophin Biosynthesis

As well as the effects on gonadotrophin secretion exercised by the ovarian steroids and GnRH previously detailed, *de novo* hormone synthesis is also modulated.

1.4.3.1. GnRH

GnRH has been shown to stimulate the synthesis of gonadotrophins (Starzec *et al* 1986; Marshall *et al* 1991; McNeilly *et al* 1991). In hypothalamus-disconnected ewes, the administration of pulsatile GnRH input restores gonadotrophin subunit mRNA to pre-disconnection levels (Hamernik and Nett 1988; Mercer *et al* 1988). In castrated rats, varying GnRH pulse frequency regimes regulate the gonadotrophin mRNAs to different degrees (Papavasiliou *et al* 1986; Haisenleder *et al* 1988b; Dalkin *et al* 1989). A short GnRH interpulse interval of around 8mins preferentially increases levels of α subunit mRNA, by its action on the promoter (Kay *et al* 1994), whilst 30min pulse intervals resulted in nonspecific increases of all subunit mRNA species. The longest pulse interval of 120min only increased the FSHβ mRNA. Similar results have been reported in the ewe (Leung *et al* 1988). Pulse amplitude also appears to regulate gonadotrophin mRNA levels. Following the administration of pulsatile GnRH to perifused rat pituitary cells, gonadotrophin α subunit mRNA increased 30% and 40% after 24h of 35 and 70pg/ml GnRH pulses whilst LHβ mRNA only displayed a significant increase (36%) after 24h treatment with 35pg/ml GnRH pulses. The levels of FSHβ mRNA appeared the most sensitive to GnRH pulse amplitude increasing by 84% after 24h of 70pg/ml GnRH pulses (Haisenleder *et al* 1993). In intact rats, the administration of GnRH agonist produced a 300% increase in α subunit mRNA levels with β subunit mRNA unaffected (Simard *et al* 1988). If α subunit expression is directly linked to free α secretion, this observation may be related to the agonist and not GnRH itself. It has recently been shown that GnRH agonist treatment led to initial stimulation of gonadotrophin secretion followed by suppressed secretion of intact gonadotrophins but not free α secretion (Fluker *et al* 1994). Treatment of rats with GnRH antagonists reverses the rise in gonadotrophin mRNA post-gonadectomy (Wierman *et al* 1989; Clayton 1993). In contrast, the constant infusion of GnRH to the castrate rat prevents LHβ and FSHβ mRNAs from rising but does not affect α subunit gene expression (Lalloz *et al* 1988; Rodin *et al* 1989). It may be that this lack of influence on the control of α subunit mRNA reflects incomplete gonadotroph desensitization by GnRH thus allowing differential control to be achieved. In the ewe, chronic GnRH agonist treatment, in the follicular phase, caused a decrease in LHβ and FSHβ mRNAs to 5 and 30% of luteal

control values respectively whilst gonadotrophin α subunit mRNA increased by 75% above controls (McNeilly *et al* 1991). It has therefore been suggested that, in the sheep GnRH can, under certain circumstances, negatively regulate α subunit gene expression (McNeilly *et al* 1991). The positive regulation of gonadotrophin gene expression by GnRH may be mediated via protein kinase C (Andrews *et al* 1988; Starzec *et al* 1990) with the interaction of a cAMP response element in the 5' region of the LHβ gene (Clayton 1993). The overall regulatory effect may result from the combination of increased transcription together with increased mRNA stability, as a consequence of altering the length of the respective RNAs (Clayton 1993).

1.4.3.2. Oestradiol

The ovarian steroid oestradiol both inhibits and stimulates gonadotrophin gene expression, apparently in tandem with the secretory regulation previously described (see section **1.3.1.1.**).

In ovariectomized ewes and rats, oestradiol depresses levels of α and LHβ mRNA (Nilson *et al* 1983; Gharib *et al* 1986; Gharib *et al* 1987; Clayton 1993). *In vitro* studies using human pituitary cells have shown that oestradiol also depresses FSHβ mRNA (Couzinet and Schaison 1993). Nuclear run-off assays in which the ability of isolated nuclei to transcribe mRNA is measured, indicate that oestradiol effects are attributable to altered transcription rates as opposed to changes in mRNA stability (Shupnik *et al* 1988). It is probable that the negative oestradiol effect is mediated through the hypothalamus, and hence GnRH secretion, since oestradiol treatment of cultured rat pituitary fragments does not affect α and FSHβ steady state levels but increases LHβ mRNA (Shupnik *et al* 1989c). Oestradiol administration produces a lowering of the GnRH *in situ* hybridization signal in rat hypothalamic tissue sections (Wray *et al* 1989) suggesting a decrease in GnRH synthesis and therefore a decrease in the amount of releasable GnRH. Indeed, treatment of ovariectomized ewes with oestradiol has been shown to decrease or completely inhibit pulsatile GnRH secretion in hypophyseal portal blood (Karsch *et al* 1993). This is in contrast to the oestradiol effects on LH secretion some of which occur at the pituitary level (see section **1.3.1.1**).

Species differences may exist in the regulatory mechanisms of gonadotrophin synthesis. Despite considerable evidence that oestradiol acts via GnRH in the rat, hypothalamic-pituitary-disconnected ovariectomized ewes showed no alteration of LHβ mRNA levels when oestradiol was administered with pulsatile GnRH (Mercer *et al* 1988). However,

in vitro studies of cultured sheep pituitary cells indicate that oestradiol decreases transcription and the amount of translatable and steady state mRNAs for α subunit and FSHβ (Alexander and Miller 1982; Hall and Miller 1986; Phillips *et al* 1988).

Positive oestradiol mediation of LH synthesis may be physiologically significant during the preovulatory LH surge. Previous investigations have demonstrated that the LH surge in ewes and female rats occurs in association with elevated LHβ mRNA levels (Landefeld and Kepa 1984b; Landefeld 1985; Zmeili *et al* 1986; Leung *et al* 1988) and the high oestradiol concentrations around this time in the cycle. These studies also showed that in the sheep, α subunit mRNA levels paralleled LHβ levels whilst FSHβ mRNA was at a nadir. In the rat, no elevation of α subunit mRNA occurred at this time but FSHβ mRNA was elevated immediately after the LH surge (Ortolano *et al* 1988). A similar increase in FSH gene expression was associated with the rebound release of FSH following the LH surge in the sheep (Brooks *et al* 1992). In agreement with the above steady state mRNA measurements, LHβ and FSHβ transcription rates in the rat follow similar patterns throughout the oestrous cycle (Shupnik *et al* 1989a).

Several investigations have suggested that these postive regulatory effects of oestradiol are directed at the level of the pituitary. Hypothalamic-pituitary-disconnected ovariectomized ewes given oestradiol produced an LH surge (Clarke and Cummins 1984). In the hypogonadal mouse, which is unable to synthesize GnRH, treatment with oestradiol increased α but not LHβ mRNA steady state levels (Saade *et al* 1989). In contrast, the increased concentrations of GnRH present in the hypothalamus of ovariectomized rats during an oestradiol induced LH surge suggests a hypothalamic component to the effect (Wise *et al* 1981). It is of note that much of this evidence assumes that translated protein is immediately released. The anomalies in the reported data may be resolved by future investigations determining the quantities of translatable mRNA and whether the nascent hormones are released immediately in response to physiological stimuli. Furthermore, the lack of conclusive proof for one site of action of oestradiol indicates that gonadotrophin biosynthesis *in vivo* may involve the interaction of a number of systems operating at different levels within the brain and pituitary gland.

1.4.3.3. Progesterone

The actions of progesterone on gonadotrophin synthesis have not been investigated to the same extent as oestradiol. Evidence for a suppressive role has been obtained from cell culture studies where the exposure of sheep pituitary cells to progesterone has resulted in decreases in both FSH secretion and FSHβ transcription (Phillips *et al* 1988), despite the fact that progesterone administration to ovariectomized ewes produced no changes in gonadotrophin gene expression (Hamernik *et al* 1987). *In vivo* studies in the rat have suggested that progesterone may have no synthetic regulatory effects on its own but may synergize with the inhibitory actions of oestradiol in suppressing LHβ mRNAs (Simard *et al* 1988). A further study has reported that progesterone administration to ovariectomized oestrogen-primed rats suppressed LHβ mRNA with no effect on FSHβ. However, in intact oestrogen-primed rats, both LHβ and FSHβ mRNAs increased following progesterone administration while treatment with 1mg of the progesterone antagonist RU486 increased LHβ mRNA and decreased FSHβ mRNA in pregnant mare serum gonadotrophin-primed immature rats with elevated LH and FSH plasma concentrations (Brann *et al* 1993). Thus it was suggested that preovulatory progesterone plays a role in the increased levels of gene expression in the rat around the time of the LH surge (Brann *et al* 1993). This conclusion is difficult to relate to the physiological situation in which progesterone is proposed to stimulate the maximal expression of gonadotrophin genes when the steroid is at its lowest concentration.

It is unclear if progesterone acts at a hypothalamic or pituitary level. In the monkey, the opiate antagonist naloxone reverses the progesterone-induced decrease in LH pulse frequency (Van Vugt *et al* 1984) whilst in the rat superfused hypothalami, GnRH secretion increases in response to progesterone administration (Kim and Ramirez 1985). A direct pituitary action cannot be excluded since, in sheep cultured pituitary cells, progesterone inhibits FSH secretion (Batra and Miller 1985).

1.4.3.4. Gonadal Peptides

Inhibin (Rivier *et al* 1985; Robertson *et al* 1985), activin (Vale *et al* 1986; Ling *et al* 1986) and follistatin (Ueno *et al* 1987) regulate FSH release from the pituitary. Synthesized by the developing follicle in response to FSH, inhibin and follistatin suppress FSH secretion whilst activin stimulates FSH release. In rat pituitary cell culture, inhibin decreased GnRH receptor numbers but had no effect on LH secretion (Wang *et al* 1988).

However, high concentrations of inhibin have been shown to decrease LH secretion *in vitro* in the rat (Farnworth *et al* 1988a and b). Inhibin has also been shown to suppress the spontaneous release of FSH from bovine pituitary cells (Lussier *et al* 1993) and ovine pituitary cells in culture (Tsonis *et al* 1986). These effects are paralleled in the regulation of biosynthesis: inhibin and follistatin suppress FSHβ mRNA levels in rats (Carroll *et al* 1989; Attardi *et al* 1989; Attardi *et al* 1992) and sheep (Mercer *et al* 1987; Brooks *et al* 1992) while activin addition to cultured rat pituitary cells leads to an increased level of FSHβ mRNA (Carroll *et al* 1989). *In vitro* studies on sheep pituitary cells showed that inhibin treatment causes a increase in GnRH receptor number (Laws *et al* 1990) which may contribute to the ability of inhibin to induce LH release in this species (Huang and Miller 1984; Muttukrishna and Knight 1990; Wrathall *et al* 1990). None of these peptides appear to effect the rate of LH biosynthesis.

1.5. The Granins

The granin family of proteins share a ubiquitous distribution throughout the neuroendocrine system (Wiedenmann and Huttner 1989). The first granin was described almost 30 years ago as the major secretory protein of the adrenal medulla (Helle 1966; Blaschko *et al* 1967). To date, six granins have been described and are listed with structural references as follows:

1. Chromogranin A (CgA) - cow (Benedum *et al* 1986, Iacangelo *et al* 1986 and Ahn *et al* 1987), human (Konecki *et al* 1987, Helman *et al* 1988, Mouland *et al* 1994), rat (Iacangelo *et al* 1988a), pig (Iacangelo *et al* 1988b), ostrich (Lazure *et al* 1990), mouse (Wu *et al* 1991).
2. Chromogranin B (CgB) - human (Benedum *et al* 1987), rat (Forss-Petter *et al* 1989).
3. Secretogranin II (SgII) - rat and human (Gerdes *et al* 1988, 1989), cow (Fischer-Colbrie *et al* 1990).
4. Secretogranin III (SgIII) - rat (Ottiger *et al* 1990).
5. Secretogranin IV (SgIV) - structure not determined.
6. Secretogranin V (SgV) - human (Martens 1988).

NOTES

1. Classification after Huttner *et al* (1991).
2. SgIV is also known as the HISL-19 antigen.
3. SgV is also known as the 7B2 protein.

The above listed proteins encompass the entire granin family thus far characterised. Only CgA, CgB and SgII will be dealt with in detail in this review since information on the other granins is lacking from the literature. The structure of the granins is highly conserved across species boundaries. Although complete data is not available from the same species, CgA and CgB genes may reside on different chromosomes: CgA is present on chromosome 14 in the human (Murray *et al* 1987; Modi *et al* 1989, Billingsley *et al* 1994), chromosome 12 in the mouse and chromosome 6 in the rat (Simon-Chazottes *et al* 1993); CgB is localised to chromosome 2 in the mouse (Jenkins *et al* 1991). The CgA gene in the cow spans around 13.6 kb (Iacangelo *et al* 1991), with the mouse extending to 11kb (Wu *et al* 1991). In the humans, CgA spans 15kb (Mouland *et al* 1994). In these three species, the gene is composed of 8 exons and 7 introns.

The molecular weights of mature CgA, CgB and SgII are 48000, 71500 and 68000, with the mRNA lengths being single transcripts of 2.1kb, 2.6kb and 2.5kb respectively (see structural references above). In the rat, a second transcript of 1.7kb has recently been described for SgII mRNA (Kakar *et al* 1993b).

1.5.1. Structure and Biochemical Properties

The granins are hydrophilic proteins with a high charged, acidic amino acid content (Grimes *et al* 1987, Robberecht *et al* 1993). Due to these properties, CgA, CgB and SgII remain soluble after boiling (Huttner and Lee 1982; Rosa *et al* 1985). The family possess low isoelectric points of between 4.9 (CgA) to 5.6 (SgIV) (Huttner *et al* 1991) as a consequence of the phosphorylation, sialylation and sulphation reactions which modify the proteins post-translationally (Fischer-Colbrie *et al* 1982; Kiang *et al* 1982; Lee and Huttner 1983; Rosa *et al* 1985).

CgA and CgB are structurally more similar to one another than to SgII due to the presence of a homologous disulphide-bonded loop structure in the N-terminal region and sequence homology at the C-terminus (Benedum *et al* 1987). Although the C-terminal homology is also present in SgII the loop structure is absent due to the fact that SgII lacks cysteine residues (Gerdes *et al* 1988, 1989). All granins have multiple putative dibasic cleavage sites, are able to bind calcium (Reiffen and Gratzl 1986a and b; Gorr *et al* 1988; Cozzi and Zannini 1988) and are subject to calcium-induced aggregation (Gorr *et al* 1988; Gerdes *et al* 1989). The tertiary molecular structure for CgA has been determined. The protein is believed to possess a predominantly random coiled

structure, around 60%, with the remainder being composed of alpha helices (Yoo *et al* 1990). Beta sheets were absent. When the pH was lowered from 7.5 to 5.5, the coiled structural component of CgA decreased with a compensatory increase in alpha helicity (Yoo and Ferretti 1993).

1.5.2. Tissue Localisation

The granins are present throughout the cells of the neuroendocrine system including the pituitary (Eiden *et al* 1987; Fischer-Colbrie *et al* 1987; Huttner *et al* 1991). At a subcellular level, the granins are localised within the matrix of cytoplasmic secretory granules (Rosa *et al* 1985; Lloyd *et al* 1985; Hagn *et al* 1986; Fischer-Colbrie *et al* 1987; Watanabe *et al* 1991). This localisation has been used clinically to determine the cellular origin of neoplasms within the endocrine system (Lloyd *et al* 1992; Jin *et al* 1993). Plasma levels of CgA are elevated in patients with peptide secreting neoplasms (O'Connor and Deftos 1986). However, a recent report indicated that serum CgA was a poor diagnostic indicator of pheochromocytoma when renal function was impaired (Canale and Bravo 1994), whilst levels of CgA in urine were less informative than plasma concentrations for assessment of carcinoid tumour growth (Stridsberg *et al* 1993). Immunohistochemical sections which were immunopositive for CgA gave a poor prognosis for colorectal adenocarcinomas (de-Bruine *et al* 1993). Extensive studies on the distribution of both native CgA, CgB and SgII proteins and mRNAs in neoplastic and normal tissues have been reported (review: Weiler *et al* 1988; Wiedenmann and Huttner 1989; Lloyd *et al* 1989; Mahata *et al* 1991; Deftos 1991). Recently, SgIV immunoreactivity was demonstrated in human medullary thyroid carcinomas (Neuhold and Ullrich 1993) and human pituitary adenomas (Lloyd and Jin 1994). The presence of CgA, CgB, SgII, SgIII and SgV mRNA transcripts in human pituitary adenomas has also been demonstrated (Lloyd and Jin 1994).

1.5.3. Post Translational Cleavage Products

As previously stated, the structure of the granins is conducive to a high degree of post translational processing. At present, cleavage products have only been characterised for CgA, CgB and SgII. In the following sections, the enzymes known to regulate individual cleavage reactions have been identified in connection with their specific cleavage product. Although their exact role has not yet been exactly defined, two kex2-related proteases known as PC1 and PC2 have been localised to the chromaffin granule membrane (Christie *et al* 1991) and in vesicles in the bovine neurohypophysis

colocalised with secretoneurin, a cleavage product of SgII (Egger *et al* 1994). Previously, it has been demonstrated that these enzymes participate in prohormone processing (Seidah *et al* 1990; Thomas *et al* 1991). Thus, they may participate in the post-translational cleavage of a number of the granins.

1.5.3.1. Chromogranin A

The processing of the CgA peptide in the pancreas by cleavage at dibasic residue 131 by a carboxypeptidase yields an N-terminal fragment known as betagranin (Hutton *et al* 1987). An analogue is present in chromaffin granules (Helle *et al* 1990). CgA may also be cleaved to form a 49 amino acid fragment known as pancreastatin. Cleavage is effected by an endopeptidase and is increased at a low pH, suggesting that differing ionic environments throughout the cell may regulate the process (Arden *et al* 1994). Pancreastatin has been shown to inhibit the stimulated secretion of insulin from the β cells in the Islets of Langerhans in the endocrine pancreas (Tatemoto *et al* 1986; Efendic *et al* 1987). The fragment also negatively regulates transcription rates from CgA and parathyroid hormone genes whilst decreasing the stability of the respective mRNA species (Zhang *et al* 1994). Parastatin (porcine CgA amino acids 347-419) inhibits parathyroid cell secretion (Fasciotto *et al* 1993). When the parathyroid gland secretes parathyroid hormone, three CgA N-terminal fragments of 24, 26 and 33kd are co-released with secretion of the 26kd fragment increased in response to low calcium incubation conditions (Drees and Hamilton 1994). Intravenous pancreastatin administration in laparotomized rats decreased plasma epinephrine concentrations, indicating a role in the modulation of adrenal medulla secretion (Sanchez-Margalet and Goberna 1993). The vasostatins, comprising the CgA N-terminus, and chromostatin (amino acids 124-143) inhibit contraction in arterial and venous walls (Aardal *et al* 1993a, b). Following neural stimulation of the chromaffin cells in perfused bovine adrenal glands, CgA cleavage products including vasostatins were released (Helle *et al* 1993). Chromostatin inhibits catecholamine secretion from chromaffin cells (Simon *et al* 1988; Galindo *et al* 1991). Intact CgA applied to perfused rat retina inhibits potassium induced dopamine release (Gibson and Munoz 1993). The N-terminal 40 amino acids of CgA act as a releasing factor for calcitonin gene related product (CGRP) whilst preventing the secretion of calcitonin and parathyroid hormone related protein from cells in culture (Deftos *et al* 1989; Deftos *et al* 1990; Drees *et al* 1991). Finally, data from cultured AtT-20 mouse cells indicates that CgA or one of its fragments inhibits pro-opiomelanocortin secretion (Wand *et al* 1991).

1.5.3.2. Chromogranin B

CgB is known to cleave to form 6 products in different species: human GAWK (amino acids 420-493) and CCB (597-657); bovine BAM-1745 (547-560); ovine OA 12, OA 8, OA 60 (Benjannet *et al* 1987; Flanagan *et al* 1990; Lewis *et al* 1984; Micanovic *et al* 1985). No functions have yet been attributed to these peptides.

1.5.3.3. Secretogranin II

The proteolytic processing of SgII produces a number of fragments including SgII residues 154-186 known as secretoneurin (Sn) (Kirchmair *et al* 1993). This product is distributed throughout the brain with high areas of immunoreactive nerve fibres concentrated in the lateral septum, the medial region of the amygdala, medial thalamic nuclei, the hypothalamus, habenula, nucleus interpeduncularis, locus coeruleus, nucleus tractus solitarius, the substantiae gelatinosae of the caudal trigeminal nucleus and of the spinal cord (Marksteiner *et al* 1993a). In the human forebrain, Sn appears to share a similar distribution in many areas to substance P (Marksteiner *et al* 1993b). An immunologically distinct form of SgII may be present in the pituitary, although the C- and N-termini and the molecular weight appear identical to SgII (Conn *et al* 1992).

The application of Sn to perifused rat striatal slices stimulates dopamine release (Saria *et al* 1993). Sn also causes human monocytes to migrate *in vivo* and *in vitro* (Reinisch *et al* 1993).

1.5.4. Control of Granin Synthesis and Release

1.5.4.1. Control by Hormones Associated With Reproduction

In the context of the reproductive cycle, perhaps the most important observation relating to granin biosynthesis is the regulatory role apparently exercised by oestradiol. In female rats, a significant ($P<0.01$) decrease in CgA pituitary content was observed between animals aged 14 days and 60 days (Anouar and Duval 1991) which correlated with increased oestradiol levels. The post-ovariectomy increase in CgA and its mRNA may be reversed by oestradiol treatment (Anouar *et al* 1991a and b). In contrast to the

female, the male rat pituitary contains higher CgA levels (Fischer-Colbrie *et al* 1992). It has been suggested that the increased CgA mRNA abundance is due to the lower oestradiol concentrations present in the male (Fischer-Colbrie *et al* 1992), but no investigations on the potential effects of high testosterone have been reported. The mechanism of the oestradiol action is not known: oestradiol does not alter steady state CgA mRNA in the adrenal medulla (Anouar *et al* 1991a; Fischer-Colbrie *et al* 1992). The action may be targeted directly at the pituitary as oestradiol treatment of rat cultured pituitary cells produces a decrease in CgA mRNA and CgA protein content of the cells (Anouar and Duval 1992).

The effects of oestradiol on SgII throughout the oestrous cycle of the rat have been described but the results are conflicting. Although the two existent reports demonstrated that SgII was under the negative regulation of oestradiol, one account details a decrease in SgII mRNA throughout the oestrous cycle with a peak at oestrus (Kakar *et al* 1993b), whilst the other indicates the lowest SgII mRNA levels occur at oestrus with the peak value during di-oestrus (Anouar and Duval 1991).

The presence of GnRH increases SgII mRNA in rats (Kakar *et al* 1993b). *In vitro* a single dose of 10^{-7}M GnRH increased CgB mRNA but not CgA mRNA in cultured CgA-positive pituitary adenomas (Song *et al* 1990).

As Sn, a cleavage product of SgII, inhibits dopamine release (Saria *et al* 1993) and dopamine is known to inhibit release of prolactin, a hormone necessary for lactation, a recent study investigated SgII expression during lactation. During lactation in rats, SgII mRNA levels, as determined by *in situ* hybridization, were elevated in the magnocellular neurones of the paraventricular and supraoptic nuclei with a greater increase observed after adrenalectomy (Mahata *et al* 1993). The increased levels of SgII mRNA may lead to increased transcription of SgII protein and hence an increase in Sn. The inhibitory action of Sn on dopamine release may assist in the removal of the inhibitory tone on prolactin, thus facilitating its secretion to induce lactation.

1.5.4.2. Control by Corticosteroids and Histamine

Following hypophysectomy, decreased levels of CgA in the adrenal medulla can be restored to pretreatment values by the administration of cortisone (Fischer-Colbrie *et al* 1988). This stimulatory role of corticosteroids was further confirmed by the fact that in the parathyroid gland, dexamethasone increases both the secretion and mRNA levels of

CgA in a calcium dependent manner (Zhang *et al* 1993). Dexamethasone also increases CgA mRNA and intact protein in the rat pituitary (Fischer-Colbrie *et al* 1989). In PC12 cells and cultured bovine chromaffin cells, increased CgA mRNA was elicited with cortisone (Rausch *et al* 1988; Iacangelo *et al* 1991). However, in the rat brain, dexamethasone has no effect on CgA mRNA or protein levels (Weiler *et al* 1990).

Levels of CgB mRNA are unaffected by cortisone (Fischer-Colbrie *et al* 1988) and oestradiol (Fischer-Colbrie *et al* 1992) *in vivo*. In contrast, cell culture experiments using GH_3B_6 pituitary cell lines, have shown CgB mRNA to be increased by oestradiol and decreased by dexamethasone (Laverriere *et al* 1991).

Finally, histamine administration to bovine chromaffin cells in culture produces a 4 fold increase in SgII mRNA without affecting the level of transcription of the other granins (Bauer *et al* 1993).

1.5.4.3. Control Mechanism

The mechanisms by which these regulators operate at the genomic level have not yet been fully defined. However, the increased amino acid incorporation into CgA observed in cultured bovine chromaffin cells in response to phorbol ester suggests that PKC may be involved (Simon *et al* 1989). The treatment of cultured bovine chromaffin cells with forskolin for more than 2 days led to a 200% increase in CgA mRNA (Iacangelo *et al* 1991), consistent with the finding of a cAMP response element on the CgA gene (Wu *et al* 1991). The exact degree of the influence of PKC is unknown as a calcitonin producing cell line increased CgA mRNA in response to phorbol ester treatment for 48h (Murray *et al* 1988), whilst the same duration of treatment to bovine chromaffin cells led to a significant decrease of CgA mRNA (Fischer-Colbrie *et al* 1990).

1.6. Scope of this Thesis

As outlined in this literature review, previous studies have demonstrated that the GnRH induced priming response in rat and mouse pituitary cells is accompanied by a series of morphological changes which result in the movement of secretory granules to the periphery of the gonadotroph prior to release. Although the priming response has been described in the sheep, no observations have been reported on any ultrastructural changes which may accompany this phenomenon. In this thesis, morphological studies

were conducted on ovine gonadotrophs in conjunction with the determination of levels of LHβ gene expression and extensive endocrinology.

Initially, an attempt was made to develop a *in vitro* perifusion system to investigate the effects of varying GnRH pulsatile inputs on LH secretion (Chapter 3). In the remainder of the thesis, *in vivo* studies in Welsh mountain ewes were carried out to determine the nature of any changes in LH granule localisation which occurred from late luteal phase through follicular phase to the preovulatory LH surge (Chapter 4). Following the LH surge, when most of the pituitary LH content is secreted, the packaging of LH into secretory granules to replenish intracellular stores was investigated in order to provide data on granule synthesis in the early luteal phase (Chapter 5). Observed morphological changes were related to LHβ gene expression throughout the cycle. In an attempt to determine if endocrine mechanisms were involved in the control of the observed changes in granule localisation, the effects of oestradiol and GnRH on secretory granule distribution were studied (Chapter 6).

The granin family of proteins have been localised histologically in the ovine pituitary. Their function is unknown. The ultrastructural localisation of CgA, CgB and SgII throughout the oestrous cycle was investigated (Chapter 7). Changes in granin gene expression were related to LHβ mRNA abundance and gonadotroph ultrastructure in an attempt to elucidate a role for the granins in the packaging and secretion of LH.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Cell Culture

2.1.1. Dispersion of Pituitary Cells

Heads from Scottish black face and texel ewes were obtained from a local slaughter house. Pituitaries were excised and immediately placed in complete medium 199 (penicillin [100I.U./ml], gentamycin [50mg/ml], amphoteracin B [0.05mg/ml], insulin [10mg/ml], 10% steroid free lamb serum, 25mM HEPES, 10mM sodium bicarbonate, 2mM L-glutamine: M199 - Life Technologies, Irvine). All subsequent procedures were carried out under sterile conditions in a BioMAT class II microbiological cabinet (Medical Air Technology Ltd, Manchester). Following removal of excess connective tissue, each gland was divided into $1mm^3$ pieces using a sterile scalpel. The pituitary cubes were washed three times in incomplete M199 (serum free) and enzymatically dispersed in a 0.1% collagenase (type I - Sigma Chemical Company, Dorset), 0.1% hyaluronidase in incomplete M199 solution at 37^0C for 90mins. The enzyme solution was removed and the tissue washed in Ca^{++}/Mg^{++}-free Hanks buffered salts solution (HBSS - Life Technologies) with 2mM ethylenediaminetetra acetic acid (EDTA - Sigma) to stop enzyme action and prevent clumping of the dispersed cells. Tissue was dispersed mechanically in HBSS using a siliconised glass pipette. The supernatant was removed, mixed 1:1 with incomplete M199 and the resultant cell suspension spun at 1500 rpm for 8 mins. The cell pellet was then resuspended in complete M199. Cell viability was assessed using the trypan blue exclusion technique with the concentration of cells being determined by counting live cells on a haemocytometer.

2.1.2. Static Cell Culture

Cells were cultured on 24 well culture dishes (Nucleon) at a density of 200 000 cells per well in a Gallenkamp CO_2 incubator (Fisons Instruments, Crawley, Sussex) at 37^0C under 5% CO_2 using complete M199. Culture media were replaced every 48h and the cells cultured for 96h prior to stimulation to allow their attachment to the culture surface.

2.1.3. Pituitary Cell Perifusion

Dispersed cells to be used in the perifusion system were cultured for 48h in M199 with cytodex microcarriers (Pharmacia Biotechnology, Uppsala, Sweden) preswollen in M199 for 24h using 0.4g microcarrier in 20mls M199. Approximately 90mg of microcarriers were added to $10x10^6$ dispersed cells. Disks of filter paper (Whatman 1, Whatman International Ltd., Maidstone) were placed in the bottom of 5ml sterile plastic syringes (Becton Dickinson, Madrid, Spain) which served as perifusion columns (Fig. 1). A 1.2mm diameter microlance (Becton Dickinson) was inserted through the syringe plunger to allow culture media to flow through the column. The cell/microcarrier suspension was loaded onto the column and allowed to sediment to a level of 2ml. A 1ml fluid head of culture media was introduced to prevent the cells drying out.

The perifusion apparatus (Fig. 2) consisted of a media reservoir from which culture media passed via polythene tubing (Watson-Marlow, Falmouth, Cornwall), through a switching unit, peristaltic pump (Watson-Marlow) to pituitary cells contained within perifusion columns stored in an HPLC oven at 37^0C. The switching unit comprised of 3-way solenoid switching valves (Lee Products Ltd, Chalfont St. Peter) driven by a timer system which allowed the introduction of pulses of a chosen challenge medium at regular intervals. A flow rate of 12ml/h was used. Orange manifold tubing (Watson-Marlow) ran through the pumping apparatus. The polythene tubing carried the media to the perifusion columns. Upon exiting the column, the perfusate was collected in 10 min samples via a LKB 2070 Ultrorac fraction collector (Wallac, Crownhill, Milton Keynes) in 2.5ml sample vials.

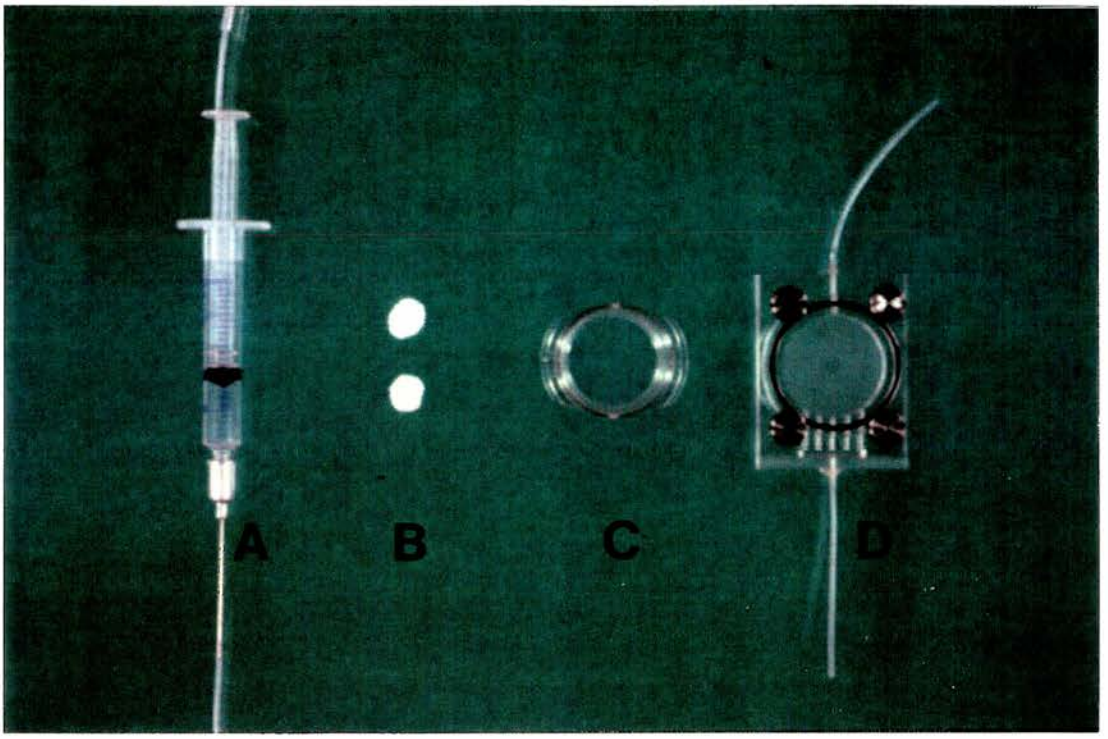
2.2. *In Vivo* Studies

2.2.1. Experimental Animals and Oestrus Synchronisation

Welsh mountain ewes aged 3-4 years, weighing 30-45kg were used in the studies. All experiments were conducted during the breeding season (October to March) at the University of Edinburgh, Marshall Building, Dryden Field Station, Midlothian. For the duration of the experiment, animals were held in individual pens or metabolism crates and were supplied with Moredun sheep nuts (Dalgety Agriculture Ltd, Bristol) and *ad libitum* water as required. Behavioural oestrus was synchronised initially by the withdrawal of intravaginal progestagen sponges (Veramix Sheep Sponge, Upjohn Ltd,

Figure 1. Cell perifusion chambers and culture membranes. During perifusions, pituitary cells attached to cytodex microcarriers were loaded into syringes which served as perifusion columns (A). Filter paper discs (B) were placed inside the syringe barrel to prevent the microcarriers from escaping. Cells were also cultured on cell culture membranes (C) and the membranes perifused in a custom designed circular chamber (D). Membranes were separated by layers of biogel.

Figure 2. Perifusion apparatus. Culture media, stored in reservoirs (A), was drawn via dialysis tubing through a switching unit (B) which allowed the introduction of pulses of GnRH or PMA. Following passage through the peristaltic pump (C), media entered the perifusion columns which were housed in an HPLC oven (D) to maintain a temperature of 37^{o}C. The perfusate was collected in 10 minute fractions using an automated fraction collector (E).


A
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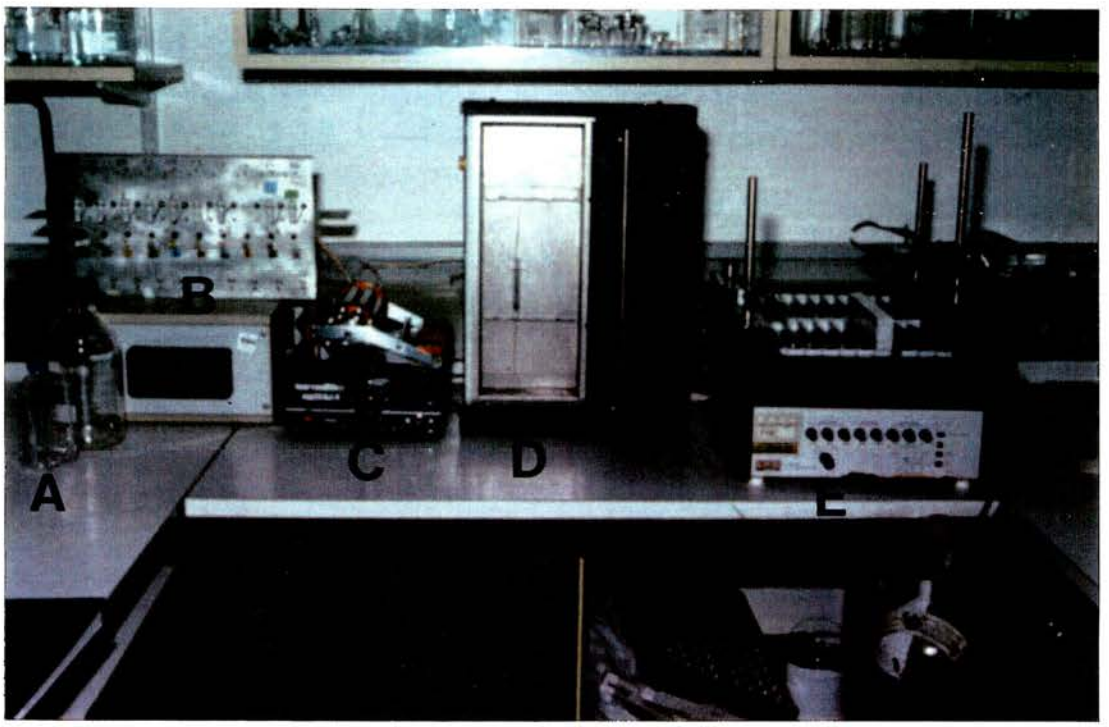
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Crawley, Sussex: 60mg medroxyprogesterone acetate per sponge) twelve days after insertion. Fourteen days later (on approximately day 10 of the following cycle), luteolysis was induced by intramuscular injection of a synthetic analogue of prostaglandin $F_{2\alpha}$ (100mg cloprostenol; Estrumate, Coopers Animal Health Ltd., Crewe, Cheshire). In experiments where the detection of behavioural oestrus was required, animals were removed from individual pens and run with a vasectomized ram at hourly intervals.

2.2.2. Blood Sampling

All blood samples were collected through a cannula (Braunula, 14G, Dunlop Veterinary Supplies, Dumfries) inserted in the jugular vein 2 to 12h before the start of blood sampling in each experiment. Each cannula was securely sutured in place and connected to a 3-way stopcock (Viggo Products, Helsingborg, Sweden) via a length of polythene manometer connecting tubing (Portex Ltd., Kent). Sterile saline (9g NaCl/l) containing heparin (20 I.U/ml - Leo Laboratories Ltd., Aylesbury, Bucks.) flushes (3ml) between blood samples maintained tube patency. Blood (2.5-4ml) was withdrawn using 5ml plastic syringes (Plastipak). The sample was then transferred to a plastic tube (Sarstedt, Leicester) containing heparinized beads (Lithium Heparin Barrier Beads, Sarstedt, Leicester) and centrifuged at 1000 rpm for 15 mins at 4^0C. The plasma fraction was decanted into 2 ml sample tubes (Teklab Medical Laboratories Ltd, Sacriston, Durham) and stored at -20^0C.

2.2.3. Radioimmunoassay

2.2.3.1. LH

Plasma concentrations of LH were determined using the radioimmunoassay described by McNeilly *et al* (1986). The iodination of ovine LH was performed using a modification of the lactoperoxidase technique (Miyachi *et al* 1972). A hormone aliquot containing 5µg of ovine LH (Reichert oLH LER 1056) was vortexed for 15 seconds with 5µl ^{125}I (Amersham, Little Chalfont), 10µl lactoperoxidase (Sigma) and 10µl H_2O_2 (1:15000, Sigma). Following the addition of 10µl cysteine-HCl (10mg/ml, Sigma) and 1ml of 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS), the mixture was vortexed for a further 15 seconds. This solution was then run on a PD10 prepacked column (Pharmacia) and 20 drop fractions collected using a fraction collector (Gilson model 203, Middleton, USA). Fractions were counted in a Mini-Assay 6-20 single tube

counter (Mini Instruments, Essex) and the three fractions around the peak of ^{125}I-oLH were pooled. Standard curves were produced with the reference standard NIH-LH-S18.

For plasma samples, R29 rabbit anti ovine LH primary antibody was used at a dilution of 1:120000. The lower detection limit of the assay was 0.3ng/ml. Plasma LH was assayed by adding 200µl of 0.1% BSA/PBS and 100µl of first antibody (rabbit anti ovine LH [R29], 1:120000 in 0.1% BSA/PBS) to 100µl of plasma sample. Following a 24h incubation at 4°C, 100µl of ^{125}I LH (15000cpm) was added and the samples incubated for a further 24h at 4°C. After the addition of 100µl second antibody (donkey anti rabbit, 1:32) and 100µl normal rabbit serum (1:800), the assay was incubated overnight at 4°C, 1ml of 0.9% saline added to each tube and the samples centrifuged at 2500rpm for 30mins at 4°C. Supernatants were decanted and the precipitate counted.

For culture media samples, R28 (rabbit anti ovine LH: McNeilly *et al* 1991) primary antibody was used at a dilution of 1:400000 in 0.1% BSA/PBS. Culture media LH was assayed by adding 200µl of 0.1% BSA/PBS, 100µl of ^{125}I-LH (15000cpm) and 100µl of diluted R28 first antibody to 100µl of culture media sample. Following a 24h incubation at 4°C, the assay protocol was the same as previously described for plasma samples. Assay sensitivity was 2.0ng/ml. The characteristics of this assay are similar to the plasma LH assay, but the assay is less sensitive due to the R28 antibody.

Pituitary LH content was determined by the method of McNeilly *et al* (1991). Pituitary glands, previously frozen in liquid nitrogen after removal from the animal, were immersed in cold 0.3M sucrose/1mM EDTA/tris HCl, pH 7.4 medium (SET) using 1ml of medium/ 100mg tissue. The preparation was homogenised for 30secs on ice using a Polytron homogeniser (Kinematica, Lucerne, Switzerland). A 0.5ml aliquot of homogenate was treated with 0.5ml of 100mM sodium carbonate containing 1% Triton X 100 (pH 9.0) and incubated at room temperature for 30mins to achieve maximum extraction of gonadotrophins (modified from McIntosh and McIntosh 1983; McNeilly *et al* 1991). The assay protocol was identical to that described for plasma LH concentrations with two exceptions.:

1. the first antibody was R28 and was used at 1:400000.
2. the first antibody and ^{125}I-oLH were added together on the first day of the assay.

2.2.3.2. Oestradiol

Plasma oestradiol concentration was determined using a modified radioimmunoassay with reagents from an MAIA oestradiol kit (Serono Diagnostics, Fleet, Hampshire, UK) after extraction. The assay protocol was kindly supplied by Dr. G. Mann. Plasma samples were pooled over 4h periods to ensure sufficient sample volume, and assayed as single points. Plasma samples were extracted by the addition of 3ml diethyl ether (BDH, Poole) to 500µl of plasma. The solution was vortexed for 5mins and the plasma layer frozen on dry ice / industrial methylated spirit (Anderson, Gibb and Wilson, Edinburgh). The solvent layer was removed, dried under nitrogen and the sample stored at -20°C until assay. Extraction efficiency was calculated by following the same procedure with 500µl plasma and 50µl of tritiated oestradiol (5000cpm, Amersham). After being dried under nitrogen, the pellet was reconstituted in 250µl of 0.1M phosphate-buffered saline pH7.2, 0.1% gelatin, 0.2% sodium azide and 0.3% EDTA (PBGS). A 50µl aliquot was counted on a LKB 1209 Rac beta liquid scintillation counter (Wallac) and the counts expressed as a percentage of the total counts added. The extraction efficiency was 62.2±1.9%.

Extracted plasma samples were reconstituted in PBGS as above. Anti-oestradiol antibody (50µl, 1:6 in PBGS; oestradiol MAIA antibody, Serono Diagnostics) was added to 250µl of reconstituted sample together with 50µl of I^{125}-oestradiol (15000cpm, Amersham) and incubated for 2h at room temperature. Following the addition of 100µl of separation reagent (goat anti rabbit gammaglobulin linked to a magnetic particle; oestradiol MAIA separation reagent, Serono Diagnostics), the mixture was left for 10mins at room temperature. After adding 1ml of 0.1M PBS, samples were centrifuged at 2500rpm at 4°C for 10mins. The supernatant was aspirated and the tubes counted. The assay sensitivity was 0.2pg/ml.

2.2.3.3. Oestradiol Binding Capacity

The immunoneutralisation of oestradiol was confirmed by determining the binding of ^{125}I-oestradiol by plasma samples at a dilution of 1:400. The assay buffer used was 0.075M phosphate buffered saline (0.9% w/v) pH 7.2 containing 1% (w/v) gelatin and 0.01% (w/v) thiomersal. Radiolabelled oestradiol (15000cpm in 100µl buffer) was added to 0.1ml diluted plasma sample and a further 0.1ml of buffer. The solution was incubated in glass tubes overnight at 4°C. Bound tracer was separated by the addition of 0.5ml dextran charcoal (0.125% dextran w/v, 1.25% charcoal w/v in buffer) and

centrifuged at 3000rpm for 15mins. The supernatant (bound fraction) was aspirated and counted. Binding was expressed as a percentage of the total counts added.

2.2.3.4. Data Collection

Assay precipitates were counted in a 1261 Multigamma γ counter (Wallac) linked directly to an Apple MacIntosh Computer. The raw data was transferred to the computer directly via the open data port and processed using the AssayZap universal assay calculator program (Elsevier-Biosoft, UK). The programme plots a standard curve using a 4-way parameter fit. The B_O and NSB values serve as estimates of the upper and lower limits of the curve whilst the parameters fit the curve through the standard values. The programme adjusts the curve by weighting individual points according to the extent they agree with the estimated fit. Thus outlying points are ignored.

2.2.4. GnRH Binding

GnRH binding activities of pituitaries were determined by the method of Bramley *et al* (1985). GnRH tracer was prepared by radiolabelling of the GnRH agonist [D-Trp^6] GnRH ethylamide with ^{125}I using the lactoperoxidase glucose oxidase method, and chromatographed on a 1x60cm Sephadex G-25 column eluted with 0.01N acetic acid-0.1% BSA (Sharpe and Fraser 1980) by Dr. T. A. Bramley. The specific activity of the ^{125}I-GnRH was determined by self-displacement assay using immature female rat pituitary homogenates and was 90mCi/mg. Pituitary glands, previously frozen in liquid nitrogen after removal from the animal, were immersed in cold 0.3M sucrose/1mM EDTA/tris HCl, pH 7.4 medium (SET) using 1ml of medium/ 100mg tissue. The preparation was homogenised for 30secs on ice using a Polytron homogeniser (Kinematica). The pituitary homogenate (75µl) was incubated with 825µl 0.5% BSA - 40mM tris/HCl pH 7.4 and 100µl of ^{125}I - GnRH (approximately 100000 counts per sample) for 4h at 4^{0}C. Bovine IgG (0.5ml of 0.5% bovine IgG in 40mM tris/HCl, pH 7.4) and 1ml of 25% polyethylene glycol (8000 Mwt.) in 40mM tris/HCl pH7.4 were added, ice-cold, to each sample and the solution mixed thoroughly. The precipitated bound hormone was separated by centrifugation at 2500rpm, 4^{0}C for 10mins. The supernatants were vacuum-aspirated and the radioactivity of the remaining pellets counted in a NE 1600 γ counter (Nuclear Enterprises, Edinburgh, Scotland). Nonspecific binding was assessed in triplicate in the presence of 10µg GnRH agonist. Specific binding was determined by the subtraction of binding activity in the presence of excess unlabelled hormone from the total binding in the absence of unlabelled hormone.

The protein content of the samples was determined by the method of Lowry *et al* (1951) and results expressed as the pg of GnRH agonist bound/mg of protein.

2.2.5. Statistical Analysis

Differences between treatment groups were assessed using the Statview 4.0 programme (Abacus Concepts, Berkeley, USA) on an Apple MacIntosh Computer. Results were analysed using one way analysis of variance (ANOVA) followed by Fishers protected least significant difference (PLSD) test where a significant ($P<0.05$) interaction was found. LH pulse frequencies were determined using the Munro pulse analysis programme (Elsevier-Biosoft, UK). The programme operates by applying 5 different threshold criteria designated the G parameters. Parameter G1 is the minimum acceptable pulse amplitude, for a pulse one sample point wide, taken as two standard deviations above the mean baseline LH concentration. G2 is the criteria for a pulse two points wide and so the series progresses to G5.

2.2.6. Anatomical Studies

2.2.6.1. Fixation

All pituitaries were removed from the animals within 5mins of death. A 1mm thick transverse section taken from the midregion of the adenohypophysis was immersion fixed for 18h at 20^0C. The specimen was constantly agitated using a Belly Dancer (Stovall Life Science Inc., Greensboro, USA) during fixation. For histological examination, the fixative was 4% paraformaldehyde (Aldrich Chemical Company, Dorset) in 0.1M PBS. A 4% paraformaldehyde/0.1% glutaraldehyde (Agar Scientific, Stansted, Essex) mixture in 0.1M sodium cacodylate buffer was used for ultrastructural studies under the electron microscope.

After fixation, the tissue was washed 3 times in 0.1M sodium cacodylate buffer (Agar Scientific) to remove any traces of fixative, and stored under fresh 0.1M sodium cacodylate buffer at 4^0C prior to infiltration and embedding.

2.2.6.2. Tissue Processing

(a). Transmission Electron Microscopy

Tissue slices fixed for ultrastructural investigation under the transmission electron microscope were chopped into $1mm^3$ blocks. The blocks were washed for 3x20mins in distilled water under constant agitation. Samples were dehydrated in graded acetones (Fisons): 50%; 70%; 90% and 100% (10 mins each) and 100% analar acetone (3x10mins) with further agitation. Specimens were initially infiltrated in Unicryl resin (British Biocell International, Cardiff, U.K.) overnight at room temperature with constant agitation. Following 4x2h in fresh Unicryl, blocks were placed in gelatin capsules (Agar Scientific), embedded in fresh Unicryl resin and heat-polymerised at 60^0C for 48h. The use of gelatin capsules led to a more complete polymerisation throughout each sample and hence a lesser degree of specimen chatter. The capsules prevented the formation of bubbles in the resin during polymerisation. These were thought to form due to the exothermic nature of the polymerisation reaction. In this respect, this particular type of capsule may have allowed any generated heat to dissipate more efficiently than other commercially available plastic capsules.

Ultrathin 60nm sections were cut on a Reichert OMU4 ultramicrotome (Wild Leitz Ltd., Milton Keynes) using Diatome diamond knives (Wild Leitz) and mounted on 200 mesh nickel grids (Gilder Grids, Grantham). Any sections which displayed chattering were repolymerised for a further 24h at 60^0C and resectioned.

(b). Scanning Electron Microscopy

Cells grown on cell culture well inserts were fixed in 3% glutaraldehyde for 3h. Following post-fixation in 1% osmium tetroxide (Johnson Mathey, Royston) in distilled water for 1h, samples were washed in distilled water for 2x30mins and dehydrated through a graded series of acetones, as described for transmission electron microscopy, prior to critical point drying in E3000 series II jumbo critical point drying apparatus (Polaron Equipment LtD., Watford Herts.) using CO_2. After they were mounted onto aluminium stubs using carbon conductive cement (Agar Scientific), each sample was coated with gold-palladium alloy, 20nm thickness, using a sputter coater SC500 (EMScope, Ashford, Kent) and viewed in a Philips SEM 505 (Philips Electron Optics, Eindhoven, Netherlands).

2.2.6.3. Immunostaining

(a). Light Microscopy

Cells cultured on cell culture membranes were attached to glass slides using superglue. The section was brought to tris histochemical buffer (0.02M Tris, 0.5M sodium chloride, 0.1% BSA pH 8.2 [all Sigma] - THB) for 10 mins. A droplet of neat, heat inactivated normal goat serum was placed on the slide and incubated for 15 mins at 20^0C . The excess serum was shaken off the slide, 100µl of diluted primary antibody (rabbit anti sheep LHβ, 1:500 in THB: NIDDK, Torrance, California, USA), placed on the section and incubated overnight at room temperature. Excess antibody was shaken off and the sections jet washed in THB. Sections were then washed thoroughly for 5x10mins in THB and agitated throughout. Addition of 100µl of secondary antibody/ 5nm gold conjugate (diluted 1:100 in THB) was followed by a 1h incubation at room temperature. The secondary antibody solution was shaken off and the section washed 5x1min in double distilled water. The area around the specimen was dried and the gold particles adhering to the section silver-enhanced with an enhancement kit (British Biocell International, Cardiff). Slides were developed for 15 mins and the reaction stopped by washing in tap water for 2 mins. Sections were counterstained in haematoxylin and mounted using Biomount (British Biocell International, Cardiff).

(b). Electron Microscopy

Post-embedding immunostaining of ultrathin sections was achieved using a modification of the method described by Watanabe *et al* (1991). Sections were incubated with 5% normal goat serum/THB for 20mins at room temperature. Following 5x5 min washes in THB, the diluted primary antibody was incubated with the specimen overnight at room temperature (dilutions: LHβ 1:800; gonadotrophin α subunit 1:100; CgA 1:100; CgB 1:100; SgII 1:100; Sn 1:100; prolactin 1:20000). After 6x10 min washes in THB, the sections were incubated with secondary antibody/gold particle conjugate (either 15 or 5nm) at a dilution of 1:100 for 1h at room temperature. Finally, sections were washed 3x10mins in THB and 3x10mins in double distilled water. They were then allowed to dry overnight, prior to staining with uranyl acetate and lead citrate in an LKB Ultrastainer 2168050 (Wild Leitz). Sections were viewed in a Phillips TEM 400 transmission electron microscope. All incubations were carried out on droplets of the respective solution placed on a sheet of parafilm. For overnight incubations, grids on droplets were contained on parafilm within glass petri dishes. Strips of paper soaked in distilled water were placed inside the dish to prevent the droplets from drying out.

Electron micrographs displaying the ultrastructural localisation of LHβ, gonadotrophin α subunit and prolactin are shown in Figures 3, 4 and 5. The distinct difference between gonadotroph and lactotroph granule structure was demonstrated. Immunostaining for gonadotrophin α subunit was confined solely to the electron-dense secretory granule profiles as was prolactin. LHβ staining was located predominantly within secretory granules. Immunoreactivity for LHβ subunit was localised outwith the secretory granules on one occasion (Chapter 5).

2.2.6.4. Stereology

The size distributions of LHβ immunopositive granules within the ovine gonadotroph were determined using the Schwartz-Saltykov diameter analysis (Underwood 1970). In order to arrive at an unbiased sample of gonadotrophs, cells were selected by means of a systematic random sampling technique modified from that reported by Lucocq (1993). Randomised tissue blocks from the mid region of the adenohypophysis were sectioned. The sections were floated onto nickel 100 mesh grids. The position of the section on the grid was therefore random. The grid bars served as the test lattice. The pattern of sampling used is depicted in Figure 6. All gonadotrophs in each of the grid squares chosen were taken for analysis. The test pattern was followed until the required number of cells had been sampled in each animal. Transmission electron micrographs of each cell were taken. Either the negatives or the actual photographs were used for analysis. Images were captured on a Sony video camera and granule profile diameters measured using the Cue 2 Morphometric Analysis Software (Olympus Optical Company, London) on an IBM computer. For investigations of granule size in the follicular phase, cells were sampled such that a minimum of 1000 granule profile diameters were measured (Chapter 4). In the analysis of gonadotrophs in early luteal phase (Chapter 5), 10-12 cells per animal were analysed and data prepared on every animal in each group as absolute numbers of granules per cell were lower.

The mathematical basis of the Schwartz-Saltykov diameter analysis is dealt with in a readable and comprehensive manner by Underwood (1970). Salient principles and the mathematical equations required will be discussed here.

The analysis is only suitable for spherical particles. The axial ratio (Fig. 7) was calculated for 100 randomly located granules and the test was performed in three animals. A value of 1 signifies a spherical structure. The calculated axial ratio was

Figure 3. Transmission electron micrograph showing the distribution of LHβ subunit using the immunogold method. The LHβ subunit, indicated by the 15nm gold particles, was distributed throughout the matrix of electron dense cytoplasmic granules (g). Immunostaining was absent from the cytoplasm. Magnification = 93000X. Scale bar = 215nm.

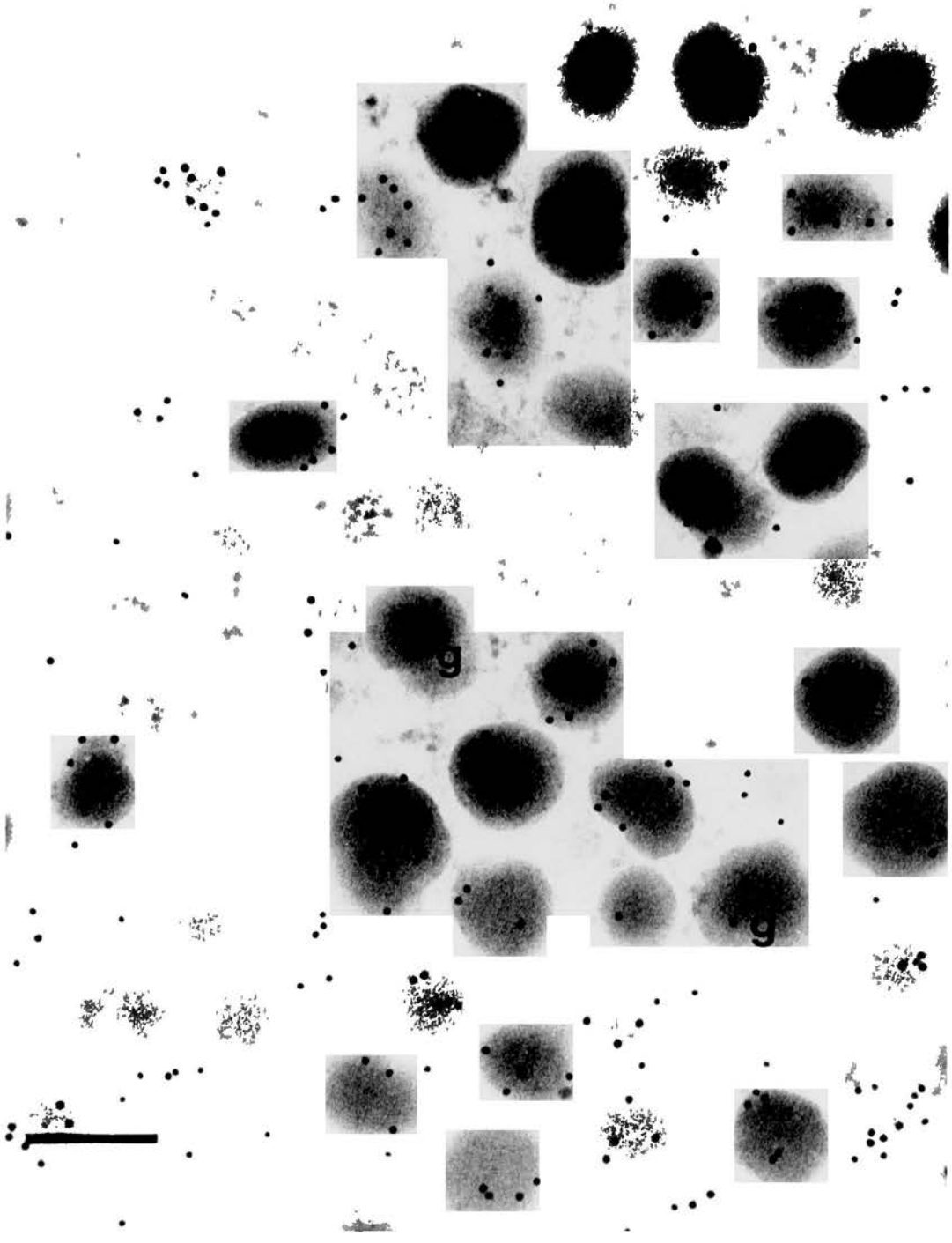


Figure 4. Transmission electron micrograph showing the distribution of gonadotrophin α subunit within the gonadotroph using the immunogold method. The α subunit, indicated by the 15nm gold particles (↟), was located within cytoplasmic granules (g). In most instances, the subunit was located towards the periphery of the granules. Magnification = 89000X. Scale bar = 225nm.

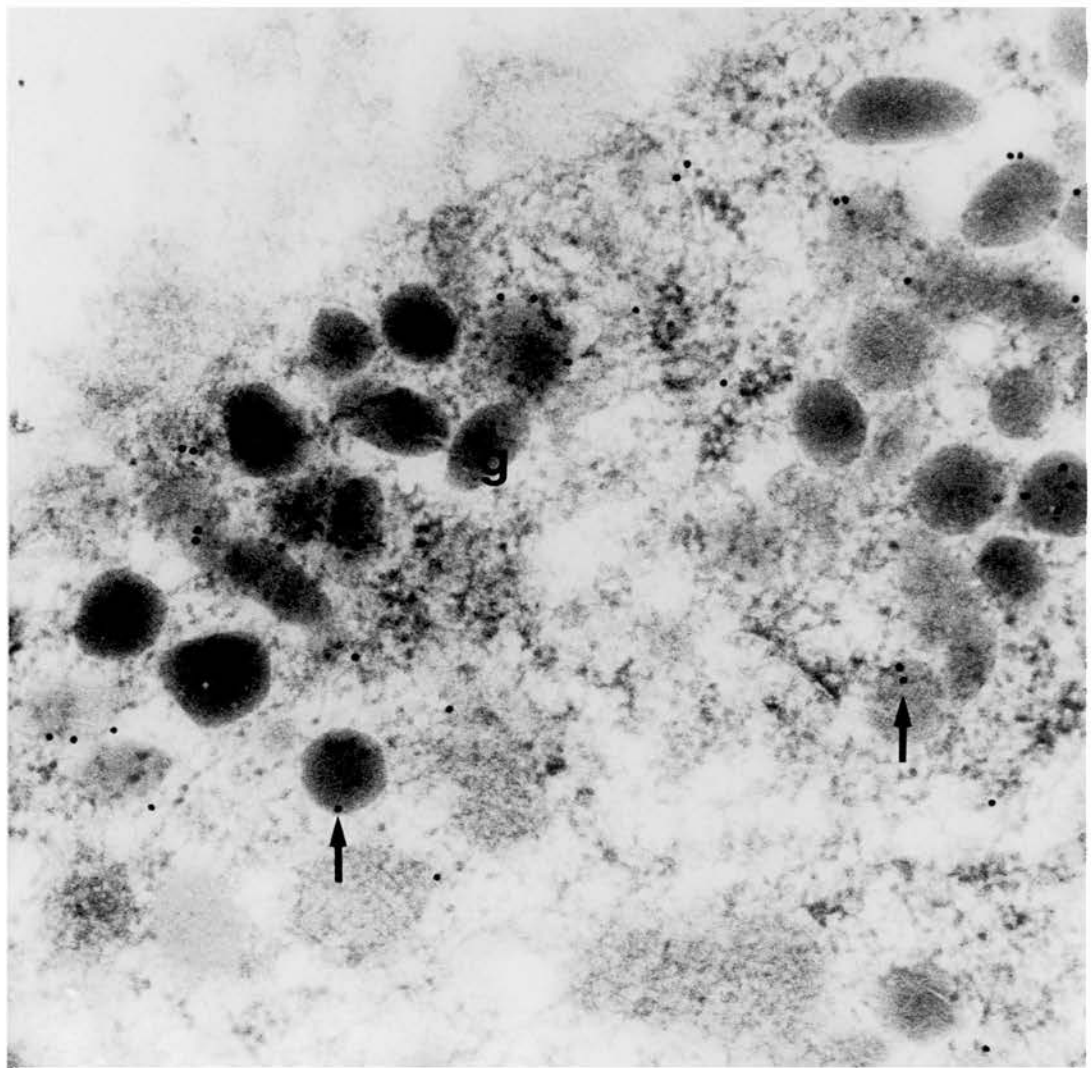
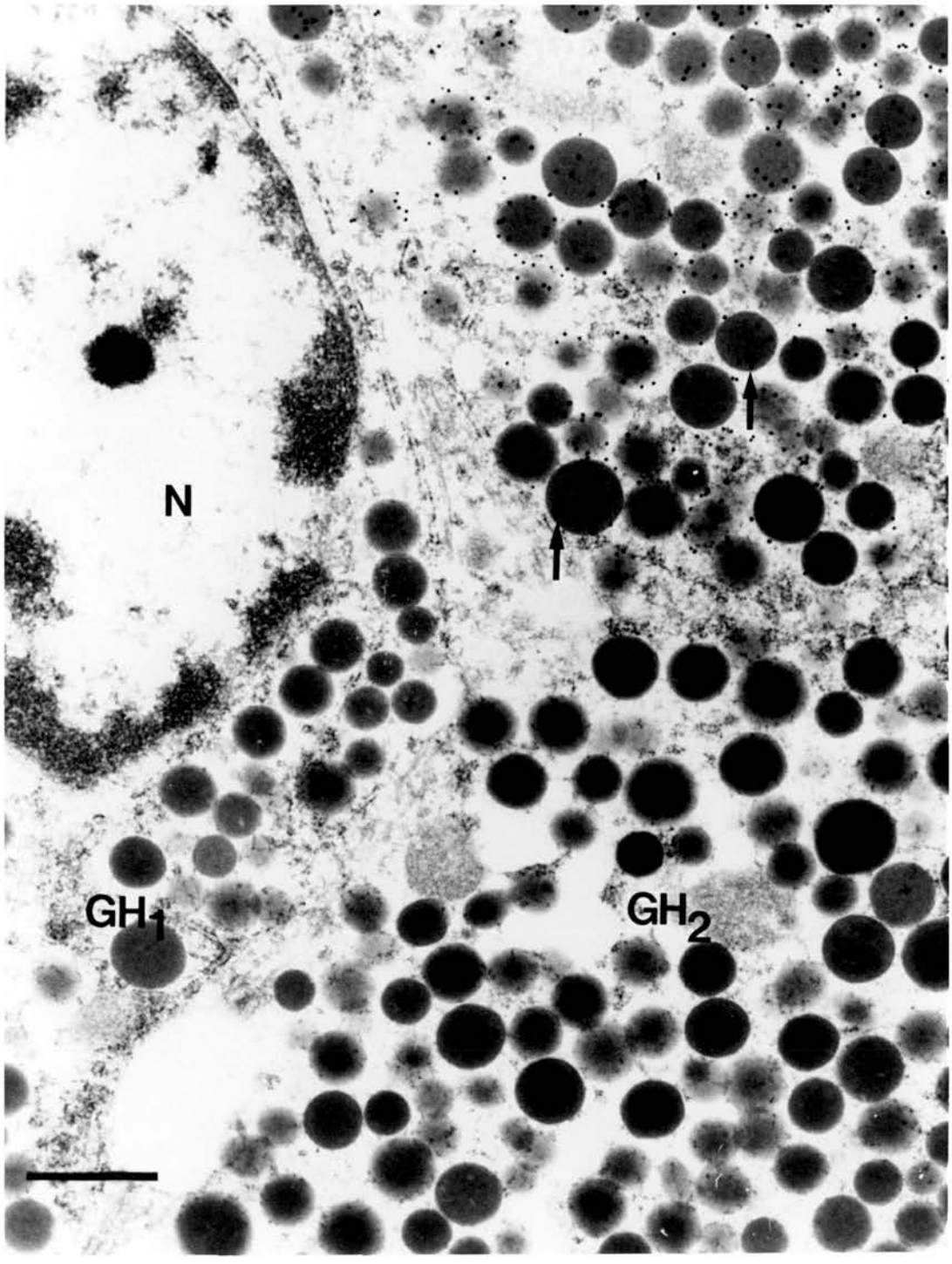


Figure 5. Transmission electron micrograph showing the distribution of prolactin within a lactotroph using the immunogold method. Prolactin, indicated by the 15nm gold particles (↟), was located within large electron dense cytoplasmic granules structurally different to the LHβ immunopositive granules present in gonadotrophs (Fig. 3). The two adjoining cells (GH1 and GH2) contained granules structurally similar to those localised within lactotrophs, but were prolactin immunonegative. In the ewe, this granule population has previously been shown to contain growth hormone (Thorpe *et al* 1990). A nuclear profile (N) was present in one of the adjoining cells. Magnification = 30000X. Scale bar = 670nm.


N
GH1
GH2

*		*		*		*
	*		*		*	
*		*		*		*
	*		*		*	
*		*		*		*

Figure 6. Test lattice used for systematic random sampling. * indicates a sampled grid square.

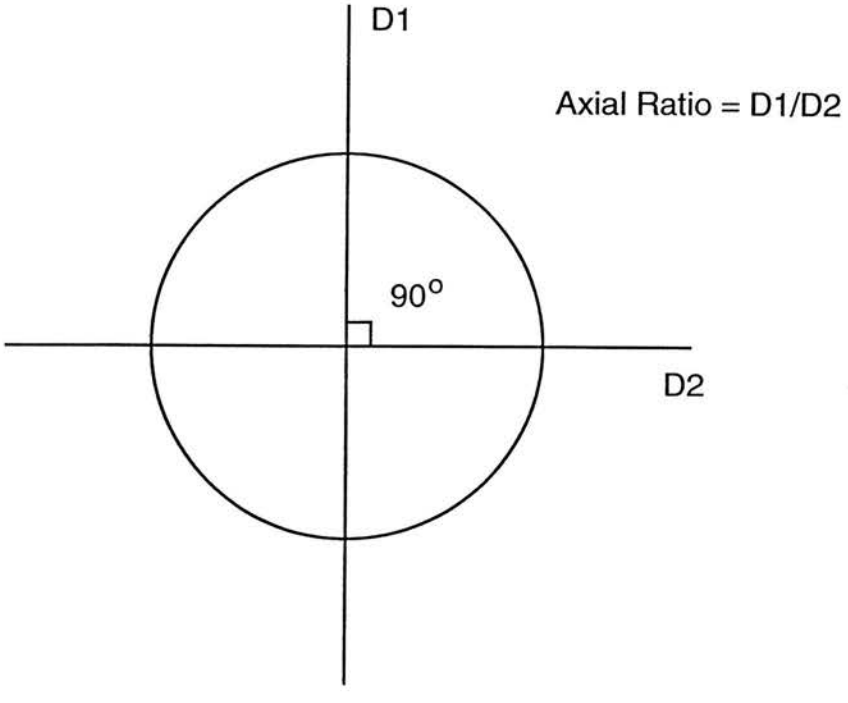


Figure 7. The spherical nature of LH granules was demonstrated by calculating the granule axial ratio. Axial ratio = D_1/D_2.

0.92±0.05 which demonstrated the near-spherical nature of the gonadotrophin granule. Granule diameter measurements were divided into 10 class intervals defined as equal percentage decrements of the maximum observed granule diameter. The observations were subclassified into each of the assigned class intervals. Thus the analysis is most applicable to a polydispersed system of spheres.

It has been determined that a monodispersed system of spheres will have 13.4% of its distribution less than or equal to half the maximum observed profile diameter ($D_{max}/2$) (Weibel and Bolender 1973). The distribution of granule profiles present in the luteal phase gonadotroph is shown in Figure 8. The percentage of the distribution with granule profiles less than or equal to $D_{max}/2$ was 48.9% indicating the polydispersed nature of the gonadotroph granules.

The working equation for the Schwartz-Saltykov diameter analysis is given in Figure 9. The coefficients were obtained from a table published in the original method (Underwood 1970) and are an assessment of the probability of a random section through a sphere resulting in a given profile diameter. The probabilities are dependent upon the number, and hence size, of class interval and the maximal observed profile radius. Figure 10 demonstrates the relationship between profile radius and the probability of intersecting a section of a set thickness. The equation combines this probability with the incident frequency in each class interval. Since the largest observed granule profiles can only come from the largest granules, the numbers of granules in the largest class were calculated first.

Clearly, observed granule profiles from the second largest class have two broad possible sources: either the actual granule diameter is that size and the granule has been sectioned through the equatorial region or the profile is the result of a section taken through a larger granule outwith the equatorial plane (Fig. 11). The number of granules in the second largest class is calculated by determining a total granule number from all sources and subtracting a likelihood that the observations came from a larger granule class sectioned outwith the equatorial plane. The calculation is then continued in a similar fashion until all the size classes are accounted for. It is possible to obtain negative values for numbers of granules in a certain class. This occurs when the probability that the observations came from larger granules outweighs the probability that granule diameter is equal to profile diameter. In these instances, the class was deemed empty.

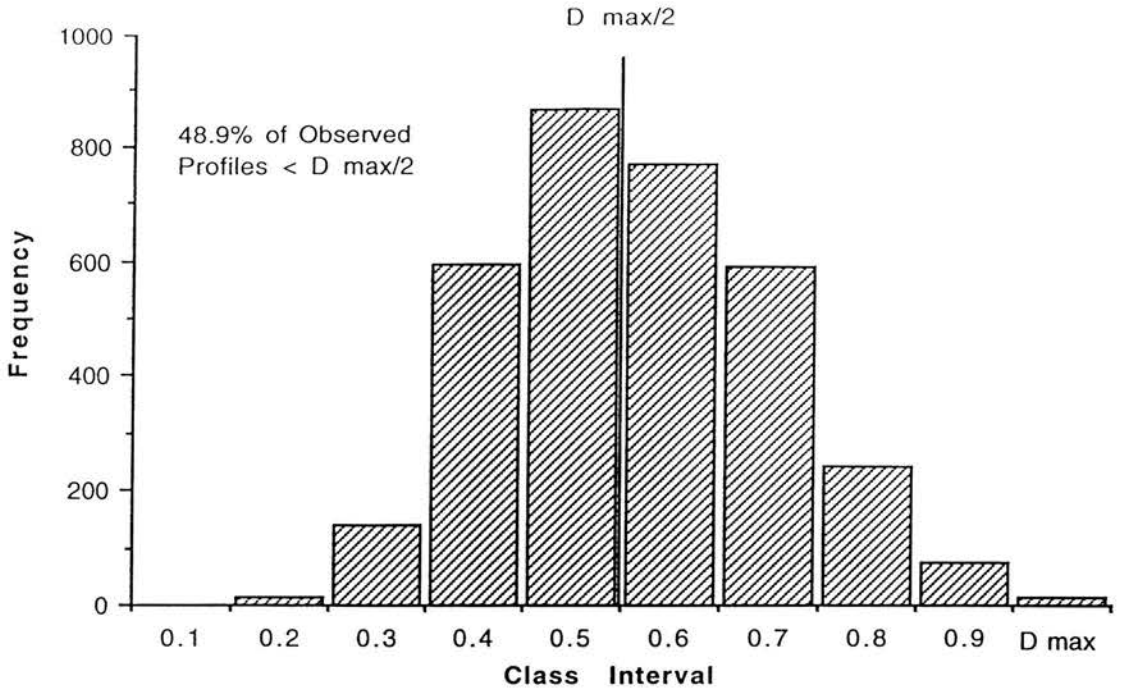
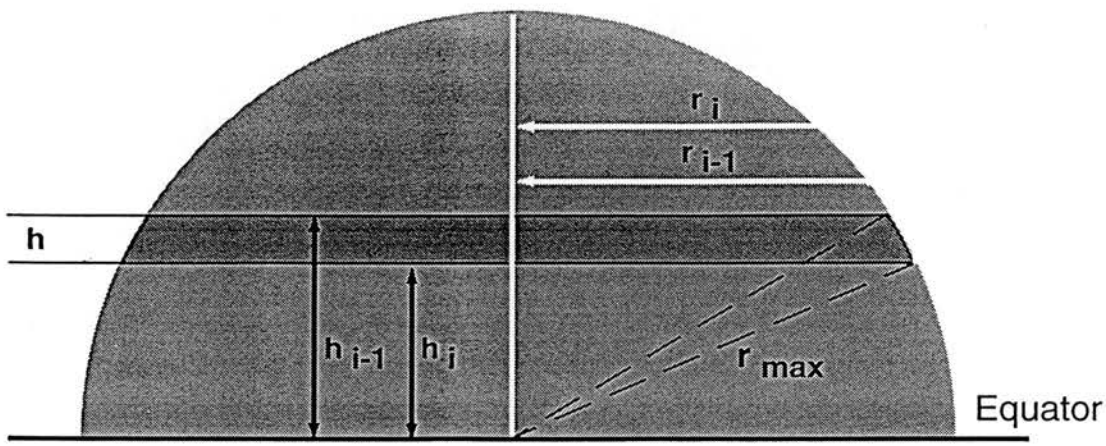


Figure 8. Frequency distribution of granule diameter class intervals from gonadotrophs in luteal phase. For a single sized granule population, 13.4% of the total distribution is less than half the maximum observed diameter ($D_{max}/2$) (Weibel and Bolender 1973). The 48.9% of observed profiles less than $D_{max}/2$ indicate that the total distribution is composed of a number of subpopulations of differing diameter.

$$(N_V)_j = 1/\Delta(\alpha_j(N_A)_i - \alpha_{i+1}(N_A)_{i+1} - \alpha_{i+2}(N_A)_{i+2} - \ldots\ldots\ldots - \alpha_k(N_A)_k)$$

$$\Delta = Dmax/k$$

Figure 9. The Schwartz-Saltykov diameter analysis equation used to calculate numbers of granules/mm^3 cytoplasm. N_V = number of particles per unit volume, a = Schwartz-Saltykov coefficient, N_A = number of observed profile diameters, k = number of class intervals, i and j = integer values from 1 to k.



$$P_{i,j} = \frac{h_{i-1} - h_i}{r_{max}}$$

$$P_{i,j} = \frac{1}{r_{max}} \left[\sqrt{(r_{max})^2 - (r_{i-1})^2} - \sqrt{(r_{max})^2 - (r_i)^2} \right]$$

Figure 10. Probability (P) of obtaining a sectional profile within a slice (class interval) of height (h). i and j = integer values from 1 to k where k = number of class intervals, r_{max} = class interval radius.

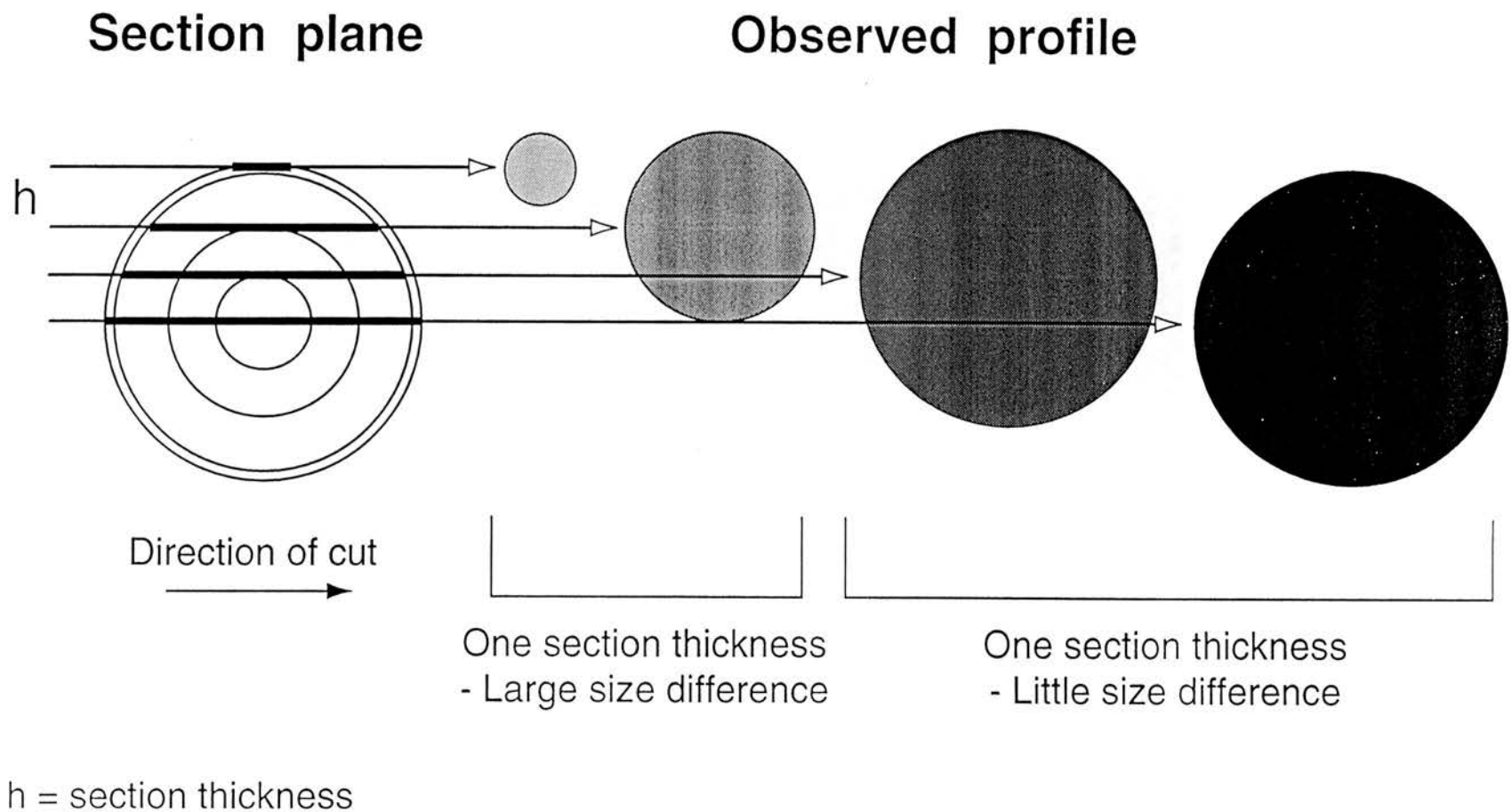


Figure 11. Relationship between plane of section and observed profile diameter. Although the probability of intersection between the granule and the section plane, at any point on the granule circumference, is the same due to the random nature of the sectioning procedure, it is more likely that the observed sectional diameter profile will be large. h = section thickness.

From the geometrical relationship between granule radius and profile radius (Fig. 10), it is apparent that the random sectioning of a monodispersed sphere population will yield predominantly larger granule profiles. This phenomenon is important when interpreting Schwartz-Saltykov plots (Chapters 4 and 5).

2.2.7. Molecular Biology

2.2.7.1. Extraction of RNA

All molecular biology grade chemicals were obtained from Sigma and IBI, Cambridge. Radiolabelled nucleotides were obtained from Amersham whilst enzymes were purchased from Boehringer Mannheim and Promega, Southampton.

The method of RNA extraction is that described by Chomczynski and Sacchi (1987). Pituitaries were removed from the animals within 5mins of death. After the removal of a slice for immunocytochemistry, the remainder of the pituitary was quartered, and opposing quarters placed in cryogenic vials (Life Technologies) and frozen in liquid nitrogen (-196^{0}C) until RNA extraction.

Frozen tissue was immersed in solution D (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarcosyl, 0.1M 2-mercaptoethanol - 1ml/100mg tissue) and homogenised using a Polytron homogeniser (Kinematica) for 30secs. Addition of 2M sodium acetate (pH4 - 0.1ml/100mg tissue) and water-saturated phenol (1ml/100mg tissue) preceded the transfer of the homogenised mixture to a sterile polypropylene tube. The suspension was mixed between additions. Chloroform/isoamyl alcohol (49:1 - 0.2ml/100mg tissue) was added and the tube shaken vigorously for 10secs until an emulsion formed. The preparation was then cooled on ice for 15mins. All centrifugations in the protocol were at 10000rpm and 4^{0}C. Centrifugation for 20mins yielded total RNA in the aqueous phase. The aqueous phase was transferred to a fresh polypropylene tube and mixed with an equal volume of cold isopropanol. The preparation was allowed to stand at -20^{0}C for 1h to precipitate the RNA. Following centrifugation for 20mins, the supernatant was removed and the RNA pellet resuspended in 0.5ml of solution D. An equal volume of cold isopropanol was added and RNA allowed to re-precipitate at -20^{0}C for 1h. A further centrifugation for 20mins produced an RNA pellet that, after careful removal of the supernatant, was washed by resuspending in 80% ethanol. After a final spin for 20mins, the total RNA was dissolved in 50-100µl of pure water containing 0.5% sodium dodecyl sulphate. RNA concentration was measured by determining the

absorbance of the sample at 260nm. The ratio of absorbances at 260nm/280nm gives a indication of the purity of the sample, a value of 2.0 being pure.

2.2.7.2. Analysis of mRNA Abundance

RNA species were separated using formaldehyde gels. Gels were prepared by dissolving 1.5% agarose (Boehringer Mannheim, Germany), 1X running buffer (10X solution contains 200mM MOPS, 10mM EDTA, 50mM sodium acetate pH 7.0) and 0.66M formaldehyde in pure water.

The gel solution, on cooling, was poured on to a gel tray (Fisons Apparatus, Coatbridge). Wells were created by placing gel combs (Fisons) into the gel and allowing it to set. Each well was loaded with 15µg of total RNA together with 16µl of RNA sample buffer, 8µl of dye solution (7.5% Ficoll, 0.1% bromophenol blue) and 1µl of ethidium bromide (1µg/ml). Each gel was run in 1X running buffer at 100V for 3-4h. RNA samples were heated for 5mins at 60^0C to denature any tertiary structure of the RNA.

The separated RNA was transferred onto a nylon membrane as follows. A piece of 3mm chromatography paper, to act as a wick, was placed on a plastic support in a pyrex glass dish. The formaldehyde gel which contained the separated RNAs, was placed on top of the wick and surrounded with parafilm on all sides. The glass dish was then filled with 20X SSC. A piece of nylon membrane (Hybond N; Amersham) cut to the exact size of the gel, after pre-wetting in pure water and 20X SSC, was laid directly onto the gel. Four pieces of 3MM chromatography paper were placed on the nylon paper followed by a pile of paper towels 4cm high. The assembly was topped with a weight (500g) and transfer left to occur overnight. Afterwards the nylon paper was removed, wrapped in cling film to keep the blot moist and the RNA fixed to the blot by means ultraviolet (UV) irradiation in a Spectrolinker XL 1000 UV crosslinker (Scotlab, Livingston). The blots were then stored at 4^0C until required for probing.

2.2.7.3. Preparation and Labelling of cDNA Probes

Double stranded DNA of CgB and SgII was prepared by amplification of the cloned cDNA insert from a plasmid vector using the polymerase chain reaction (Saiki *et al* 1988). The primers SP6 and T7 (TA cloning vector; Invitrogen, Abington, England) were used at a concentration of 0.5mM. The remainder of the PCR reaction consisted of 0.2mM dNTP (Pharmacia, Milton Keynes, England), Taq polymerase buffer

(50mM KCl, 10mM Tris/HCl pH 9.O, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100) and 2.5U Taq polymerase (Promega). Plasmid DNA (100-500ng) containing the respective cDNA insert served as the reaction template. Thirty five amplification cycles were performed in a Omni Gene Thermal Cycler (Hybaid, Teddington, Middlesex) with an annealing temperature of 45°C and a 72°C extension phase lasting 2 mins. The size of the PCR product was checked by running 10µl on a 2% agarose/TBE gel with the DNA size markers Phi X and Hind III. The amplified probe was then purified from the PCR mixture using Clontech ChromaSpin +TE-100 columns (Cambridge Bioscience, Cambridge, England) using the manufacturers instructions.

CgA DNA was prepared using the same PCR protocol outlined previously. The product was then separated by running the PCR mixture on a 2% agarose/TBE gel and the correct size fragment extracted from the gel using a spin bind DNA recovery system (FMC, Denmark) according to the manufacturers instructions.

Probes were radiolabelled with ^{32}P using a modification of the method described by Feinberg and Vogelstein (1983; 1984). The reaction was carried out using a Multiprime DNA Labelling Kit (Amersham International, Lewes, England). The probe to be labelled was dissolved to a concentration of 2-25µg/ml in double distilled water which had been UV irradiated. Following denaturation by boiling for 5mins, the probe was labelled with 50µCi of ^{32}P-[α]-dCTP in a reaction mixture composed of 5µl primer solution containing random hexanucleotides, 10mM each of dATP, dGTP and dTTP, and 10µl reaction buffer containing Tris/HCl pH 7.8, $MgCl_2$ and 2-mercaptoethanol. The reaction was catalysed by the addition of 2U Klenow enzyme and incubated for 1h at 37°C. The labelled DNA was denatured with 5M NaOH (50µl), neutralised with Tris/HCl, pH 7.6 (600µl)and 1M HCl (375µl), and added to the hybridisation solution.

2.2.7.4. Labelling of Oligonucleotide Probes

These probes were radiolabelled at the 5' end using polynucleotide kinase (PNK). Oligonucleotide (50ng) was incubated with 30µCi [γ-^{32}P]ATP, 1x kinase buffer (10 x buffer contains 0.5M Tris/HCl, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA) and 8U T4 PNK for 1h at 37°C. The labelled probe was the transferred to the hybridisation solution.

2.2.7.5. Hybridisation of Radiolabelled Probe to Membrane

All prehybridisations and hybridisations were carried out in a hybridisation oven (Hybaid, England). Nylon membranes, onto which RNA had been transferred, were prehybridised in pyrex hybridisation tubes at 65°C for 5h in buffer composed of 0.5M sodium phosphate pH 7.2, 1mM EDTA, 1% BSA, 7% SDS and 15% formamide. Radiolabelled cDNA probes were added to the hybridisation solution at a concentration of 0.5-1.0x10^6 cpm/ml and allowed to hybridise to the nylon membrane for 24-48h at 65°C.

Oligonucleotide probes were added directly to the hybridisation tube by adding 0.5ml of hybridisation solution to the labelling mixture and transferring the entire mixture with a Gilson pipetteman (Gilson, England).

2.2.7.6. Post Hybridisation Washes and Removal of Probes from Membranes

To remove excess background radioactivity, membranes were washed at 65°C, following hybridisation, using one of two protocols:

1. LHβ, CgA, CgB and SgII cDNAs were washed in 2xSSC for 1x5, 1x10 and 1x20mins.
2. the 18S oligonucleotide probe was washed in 4xSSC for 2x10mins.

To allow the reprobing of nylon membranes, initial probe hybridisations were removed by agitating with a boiling solution of 1M tris pH 7.5 (5ml), 0.5M EDTA (1ml) and double distilled H_2O (494ml) for 10mins. Finally, the membrane was rinsed with the same solution at room temperature.

2.2.7.7. Measurement of Hybridisation Signal

Following hybridisation with a radiolabelled probe, membranes were wrapped in cling film and the hybridisation signal measured in a Phosphor Imager (Molecular Dynamics, Sevenoaks, Kent). Blots probed for LHβ and 18S were exposed for 1h, whilst the CgA, CgB and SgII probings were exposed for between 3-18h. The antisense oligonucleotide for 18S ribosomal RNA (Chang *et al* 1984) was used to control for the uneven transfer of RNA onto the Northern blot. Consequently, results for mRNA abundance are expressed as the respective probe activity/18S.

Representative hybridisations of radiolabelled LHβ, CgA, CgB, SgII and 18S probes are shown in Figure 12.

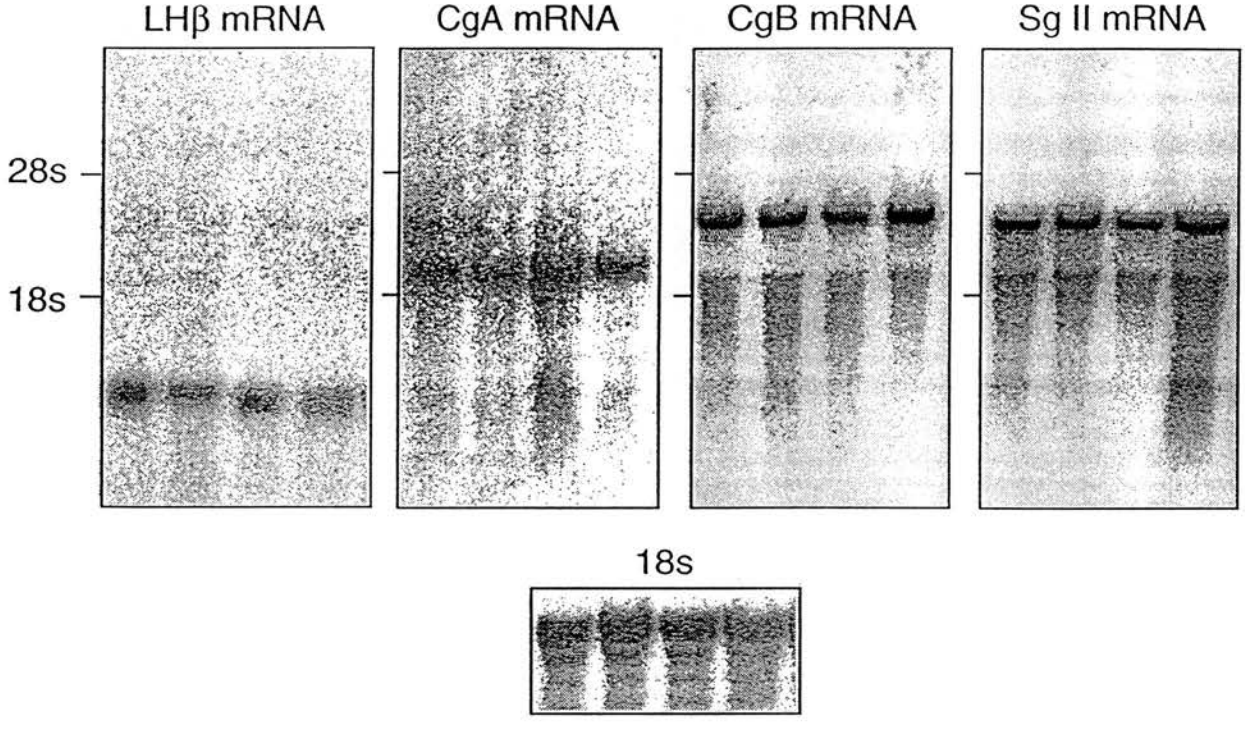


Figure 12. Representative hybridisations to Northern blots. Approximate size of mRNA transcripts: LHβ 0.7kb; CgA 2.1kb; CgB 2.6kb; SgII 2.5kb. The RNA was extracted from four separate pituitaries and 15μg loaded per lane. Images are direct print outs from a Phosphor Imager and are the result of 2h exposures.

CHAPTER 3

DEVELOPMENT OF AN *IN VITRO* PITUITARY CELL PERIFUSION SYSTEM

Introduction

Since the early 1970's, *in vitro* cell dispersion systems have been widely used to study pituitary cell function (Hopkins and Farquhar, 1973). Static cell cultures, whilst providing information regarding the possible cellular performance *in vitro,* fail to reproduce the dynamic physiological conditions experienced *in vivo*. The development of a superfusion system, duplicating the pulsatile nature of *in vivo* GnRH secretion (Smith and Vale 1980), has allowed the investigation of cell functions more closely related to those *in vivo*.

Long term perifusion of cells requires their isolation from the surrounding connective tissue. Thus, a dispersion system for sheep adenohypophyseal cells was validated and their viability determined, before progressing to the perifusion apparatus.

3.1. STATIC CULTURE

3.1.1. Validation of a dispersion system

The enzymatic dispersion of pituitary cells using trypsin appears to cause loss of the GnRH receptor and thus decrease gonadotrophin secretion in response to GnRH challenge (Dr. T.A. Bramley, personal communication). In contrast, it appears that using collagenase for cell dispersion (Huang and Miller 1980) maintains the GnRH receptor and hence would favour the maintenance of a responsive pituitary cell culture. To investigate this hypothesis, a collagenase dispersion system was developed and its performance compared to that of trypsin by means of a GnRH challenge in static culture.

3.1.2 Materials and Methods

Adult blackface and texel ewe heads were obtained from a local slaughter house. Pituitaries were removed and enzymatically dispersed using the trypsin

(Tsonis *et al* 1986) and collagenase (**2.1.1.**) methods previously described. Cell viability was assessed using the trypan blue exclusion technique and cells plated at a density of 200,000 cells per well using 24 well culture dishes (Nucleon, Roskilde, Denmark). The culture media was replaced every 48h. Cells were challenged with GnRH (Cambridge Research Biochemicals, Northwich, Cheshire) at concentrations of 10^{-9}M and 10^{-12}M for 4h at 37°C after 4, 6 and 8 days in culture. Culture media was removed and LH production measured by radioimmunoassay (**2.2.3.**). All treatments were performed in triplicate and the experiment repeated three times in total.

3.1.3. Results

Dispersions carried out using trypsin yielded 87.4±3.1% of viable cells compared to 68.2±2.3% obtained with collagenase. The cells from the collagenase dispersion were present in small chains or monolayer clumps of 5-10 cells with the occasional single cell whereas trypsinization produced, predominantly, isolated cells.

The cells dispersed using collagenase showed a significant ($P<0.05$) increase in LH secretion in response to a 10^{-9}M GnRH challenge after 4 days in culture (Fig. 13). LH concentrations increased from a control value of 93.4±8.0ng/ml to 202.1±20.6ng/ml as a result of GnRH stimulation. Following a challenge with 10^{-12}M GnRH there was no significant release of LH (98.8±9.7ng/ml). After a further two days in culture, the concentration of LH secreted by the control cells was 65.0±12.3ng/ml. Treatment with 10^{-9}M GnRH again produced a significant ($P<0.05$) stimulation of LH release to a concentration of 127.3±13.5ng/ml whilst the LH concentration of 64.2±18.8ng/ml resultant from the 10^{-12}M GnRH challenged did not differ significantly from controls. By the 8th day in culture, although a stimulatory trend was observed for LH released in control wells (51.8±11.7ng/ml) to the 10^{-9}M GnRH challenge (97.3 ± 23.5ng/ml), this did not reach a level of significance. There was no significant release of LH in response to 10^{-12}M GnRH (66.2±12.9ng/ml). In the control wells a significant ($P<0.05$) decrease was observed between LH concentrations on day 4 and day 8 in culture. A similar significant ($P<0.05$) decline was evident over the three days on which cells were challenged with 10^{-9}M GnRH. The decreasing trend of LH released from cells stimulated with 10^{-12}M GnRH at the three time points did not reach a level of significance.

In contrast, cells dispersed using trypsin (Fig. 14) digestion showed no significant differences in LH released from control cells stimulated on days 4,6 and 8

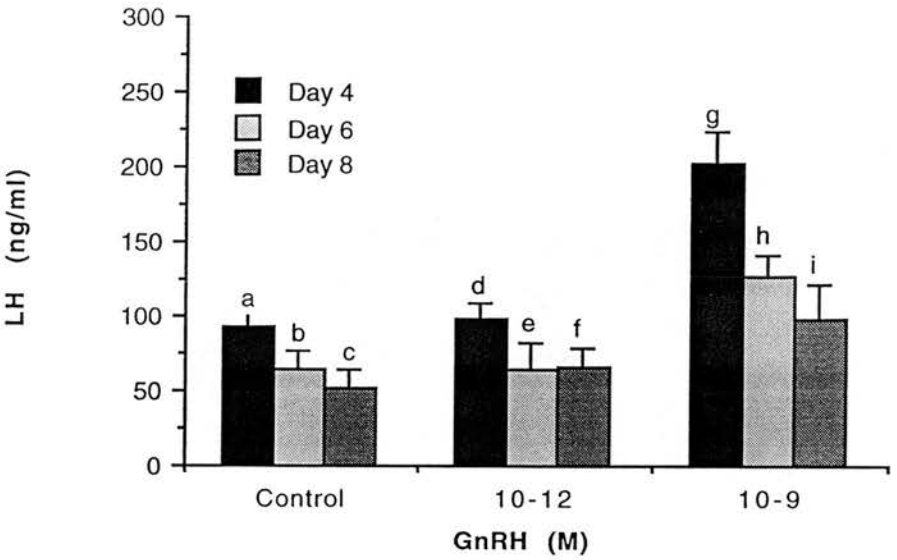


Figure 13. Static culture determination of the responsiveness of pituitary cells dispersed using collagenase to GnRH (10^{-9}M). Culture media was removed and replaced with fresh media every 48h and cells challenged with GnRH after 4, 6 and 8 days in culture. Results are given as mean ± SEM and results analysed by two-way ANOVA. Significant ($P<0.05$) differences: a, c; a, g; b, h; d, g; e, h; g, h; g, i.

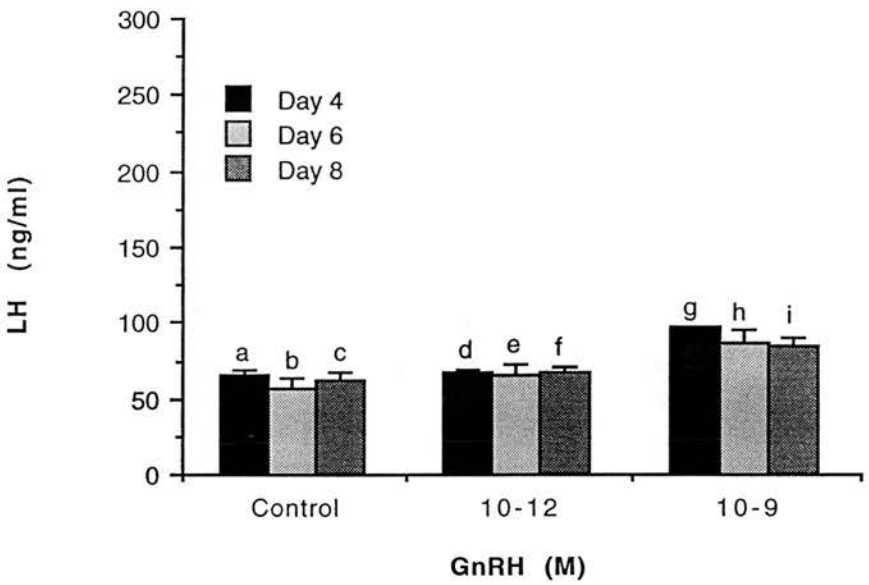


Figure 14. Static culture determination of the responsiveness of pituitary cells dispersed using trypsin to GnRH (10^{-9}M). Culture media was removed and replaced with fresh media every 48h and cells challenged with GnRH after 4, 6 and 8 days in culture. Results are given as mean ± SEM and results analysed by two-way ANOVA. Significant ($P<0.05$) differences: a, g; b, h; c, i; d, g; f, i.

(66.1±2.3ng/ml, 57.0±6.4ng/ml and 61.7±6.2ng/ml respectively) as shown in Figure 14. LH concentrations following a 10^{-12}M GnRH challenge on days 4,6 and 8 did not differ significantly from control values on each day on which the challenge was made (67.4±2.0ng/ml, 65.4±7.3ng/ml and 66.6±4.7ng/ml respectively). Finally, 10^{-9}M GnRH caused a significant ($P<0.05$) stimulation of LH secretion, releasing 96.7±0.7ng/ml on day 4, 86.3±8.5ng/ml on day 6 and 85.3±5.0ng/ml on day 8. The magnitude of the stimulation did not differ significantly between the days on which the challenge was made.

3.1.4. Discussion

Of the two dispersion methods, the cells obtained using the collagenase enzyme showed a greater release of LH following stimulation on days 4 and 6 than cells dispersed with trypsin. Although the cells produced by the trypsin method were approximately 90% viable, they performed less well than the collagenase dispersed cells with a viability of around 70%. The concentration of LH released applies to similar numbers of cells in each case as plating densities were calculated on the live cell counts obtained using a haemocytometer. Any dead cells which were added to the culture well at this time would be unable to adhere to the culture surface and would therefore be removed when the culture media was changed after the first 48h incubation. The larger standard errors, obtained from the cells dispersed using collagenase, may have resulted from a slightly greater variance in plating density. The existence of chains and small groups of cells may result in a greater inaccuracy in the haemocytometer-derived cell density due to a less even distribution throughout the dispersion medium. Alternatively, the lower percentage viability in the collagenase cells is suggestive of a greater degree of cell damage. Differences in survival rates of cells between wells would also result in a larger standard error.

The increased LH secretion obtained using the collagenase dispersion system may result from the greater preservation of the GnRH receptor through the dispersion protocol (Dr. T.A. Bramley, personal communication). Increased numbers of GnRH receptors may lead to a greater stimulatory response from a unit GnRH stimulus. Furthermore, the small clusters of cells typically produced following collagenase dispersion may be functionally superior as it has been shown that cellular organisation in the pituitary of the rat is not random (Horvath *et al* 1977, Nakane 1970). Intercellular communications by means of junctional complexes may allow the production of a more physiological response to the releasing hormone present. The collagenase dispersion technique may

preserve the anatomical relationships of small groups of cells thus increasing the functional integrity of the preparation.

Rat pituitary cell aggregates, produced by enzymatically dispersing cells with trypsin and allowing them to reaggregate by constant gyrotatory shaking, have been shown to remain responsive to GnRH after 3 weeks in culture (Van Der Schueren *et al* 1982). Junctional complexes were observed between the aggregates thus suggesting the possibility of intercellular communication between the cultured pituitary cells. This association may be functionally beneficial. In other cell types, cellular aggregation has been shown to facilitate increased functional competence. The expression of glutamine synthase, an enzyme present in neural retina tissue and regulated during development, was absent from single cell suspensions but present in multicell aggregates of retinal tissue (Linser and Moscona 1979). In reaggregated brain cells, greater enzyme activities of choline acetyltransferase and glutamate decarboxylase were observed when compared to monolayers and brain cell suspensions (Seeds 1971).

The decreasing LH concentrations released from all treatment groups as the length of the culture increased, may be due to a decrease in the amount of releasable hormone or a reduction in the sensitivity to the GnRH stimulus. It is thought that a decrease in the amount of releasable hormone is the probable cause due to the fact that the reduction was also observed in the untreated control wells. Either the LH content of all the gonadotrophs decreases simultaneously or some cells with a lower response threshold to the GnRH input may empty their LH stores more rapidly than others which could continue secreting lower amounts of LH in response to subsequent challenges. Both these mechanisms would result in an observed decrease in the 'releasable pool' of LH (Smith and Neill 1987; Neill *et al* 1987). These authors have shown that rat gonadotrophs which are non-secretory are unable to release LH following stimulation with GnRH, using a reverse haemolytic plaque assay. This may be due to a lack of GnRH receptors since increased numbers of mature secretory gonadotrophs at di oestrus show an ability to bind GnRH (Childs *et al* 1994). This suggests that both the stimulated LH secretion and the constitutive release in control wells originate predominantly from the same gonadotroph subpopulation. Cell death during the culture period, resulting in LH release may also contribute to basal levels. Due to the exhaustion of the releasable pool, the cells dispersed with collagenase may be unable to respond with a significant LH release following the third GnRH challenge on day 8 in culture. The lower concentrations of LH released from the trypsinized cells may

preserve enough releasable LH allowing a significant response to the final stimulation. These results will be discussed further in Chapter 8.

The collagenase dispersion was chosen to provide cells for perifusion due to the increased responsiveness to the GnRH challenge.

3.2. PERIFUSION OF CULTURED CELLS

3.2.1. Materials and Methods

3.2.1.1. Microcarrier Support

Cytodex microcarrier beads (Pharmacia) were swollen in complete medium 199 overnight (0.1g/20ml). Cells, enzymatically dispersed using collagenase as previously described, were incubated with microcarriers for 48h at 37°C to allow attachment of live cells. The microcarriers were then placed in the perifusion apparatus as detailed in Chapter 2. The cells were cultured for a 3 day period and pulsed with 10^{-9}M GnRH or phorbol 12-myristate, 13-acetate (PMA: Sigma Chemical Company) for 5mins at 2 hourly intervals during each day of the experiment. The cells were allowed to recover overnight with the continuous perifusion of complete medium 199. Samples were collected every 10mins during each day and gonadotrophin levels measured by radioimmunoassay.

3.2.1.2. Culture Membrane Support

Dispersed cells were cultured at a density of 1,000,000 cells/ml on cell culture well insert membranes (Falcon) in complete medium 199 at 37°C for 48h. The membranes were then removed and placed in the circular perifusion chamber (Chapter 2, Fig. 1). A total of 4 membranes, each separated by a layer of biogel (Pharmacia)[0.5g biogel swollen overnight in 10ml complete medium 199] were used in the chamber. The cells were then challenged with 10^{-9}M GnRH as previously described.

3.2.1.3. Structural Observations

Cells in the perifusion columns were fixed by pumping 3% glutaraldehyde into the column. After 2h, the fixative was removed by flushing 5 times with 0.1M sodium

cacodylate buffer. Post-fixation in 1% osmium tetroxide was achieved using the same procedure. Cell culture well inserts were immersion-fixed using similar fixatives and fixation times. Areas of the culture inserts were fixed in 4% paraformaldehyde for immunogold localisation of the LHβ subunit. Specimens for ultrastructural investigation were embedded in unicryl and sectioned for transmission electron microscopy as previously described. Culture well inserts were attached to glass slides using super glue, immunostained and examined under the light microscope. Inserts were also mounted, sputter coated and viewed under the scanning electron microscope (**2.2.6.2.** and **2.2.6.3**).

3.2.2. Results

3.2.2.1 Perifusion

In the course of initial studies to attempt to validate the perifusion system and eradicate problems, the pulsatile release of LH was demonstrated on two occasions using cells obtained from separate dispersions.

Following the flushing out of LH from dead and dying cells which were unavoidably loaded onto the column, the gonadotrophs produced well-defined LH pulses in response to the GnRH challenge (Fig. 15). On the first day, three pulses of peak amplitude 8.05, 12.12 and 12.25ng/ml were produced. On the second day of stimulation, the pulse amplitudes increased to 24.41, 20.65 and 17.42ng/ml respectively. The pulsatile response was still present on the third day of the experiment although a blocked line during the second night resulted in a high initial LH level on day 3. This was due to a flushing of residual hormone through the system when the blockage was cleared. The single pulse recorded on the final day of the perifusion had a peak amplitude of 9.77ng/ml. Between pulses, the basal concentration of LH was 3-4ng/ml.

On a separate occasion, pulsatile LH release in response to 10^{-8}M PMA was achieved over two days (Fig. 16). Pulse amplitudes on the first day were 44.49, 42.02 and 28.06ng/ml. The challenges on the second day produced further pulsatile responses, although the amplitudes decreased to 16.54, 21.95 and 20.42ng/ml respectively.

After the eradication of functional problems with the perifusion apparatus such as blockage of media lines, column leakage and mechanical failures of the fraction collector, cells from five separate dispersions were perifused using three columns per

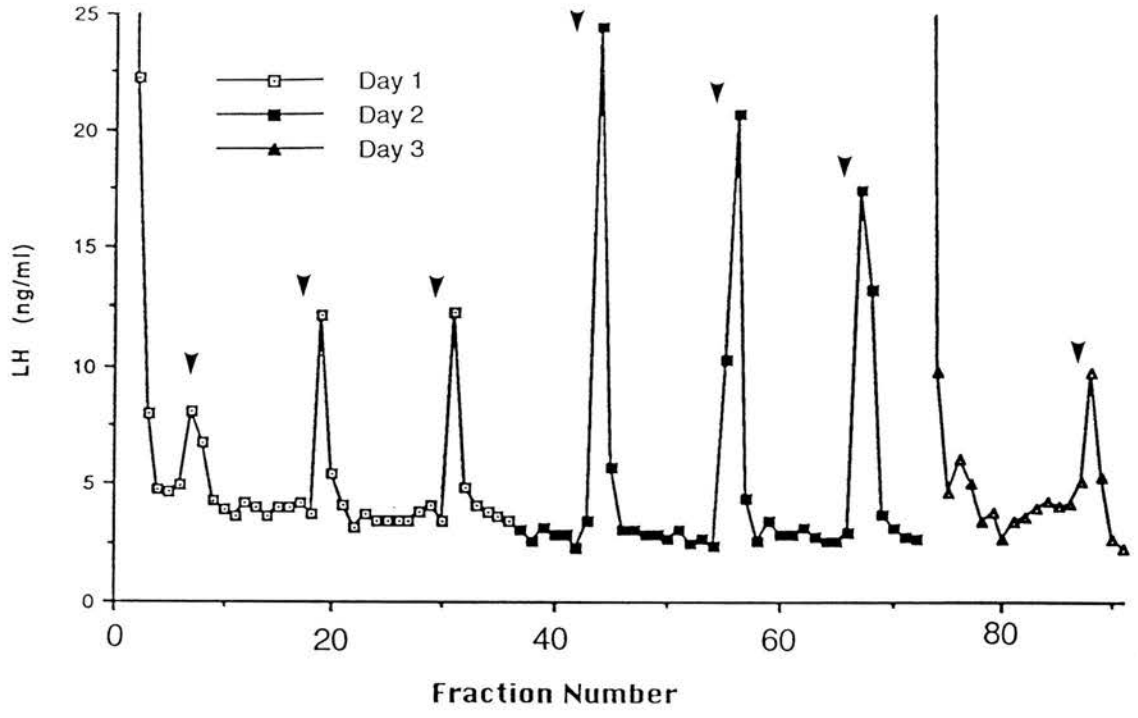


Figure 15. Pulsatile LH release from dispersed pituitary cells, using syringe columns, challenged with GnRH (10^{-9}M) over three days in perifusion cell culture. Arrowheads indicate a pulse of GnRH or PMA. The break in fraction collection between days 2 and 3 was due to a blockage in the peristaltic tubing of the perifusion apparatus.

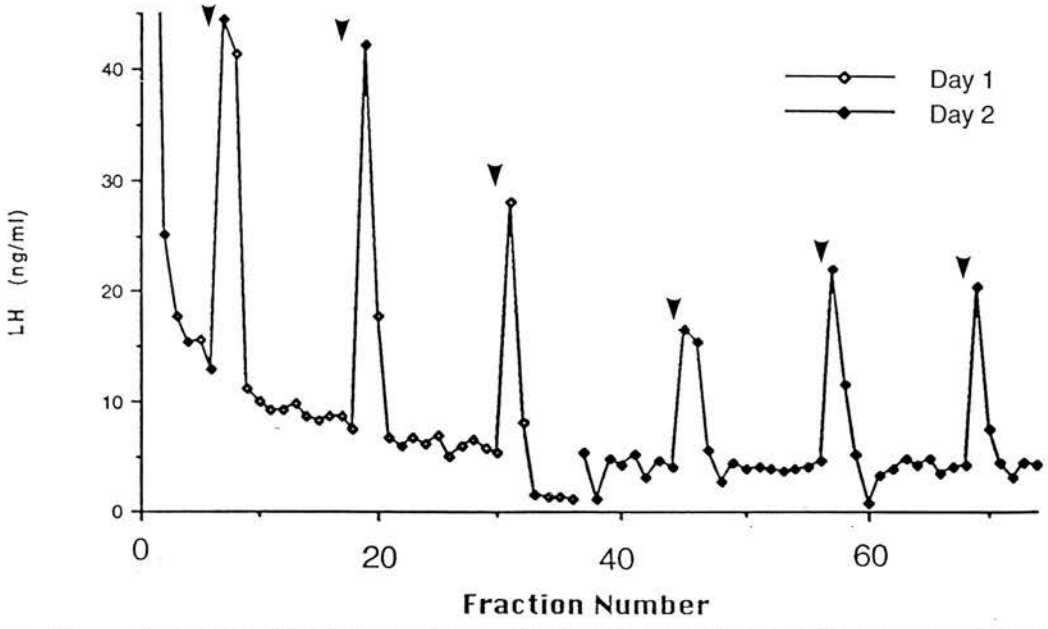


Figure 16. Pulsatile LH release from dispersed pituitary cells, using syringe columns, challenged with PMA (10^{-8}M) over two days in perifusion cell culture. Arrowheads indicate a pulse of PMA.

dispersion. These repetitions of the experiment failed to produce any stimulated pulsatile LH release despite the mechanical integrity of the culture system. Representative examples, from separate dispersions, of media LH profiles are shown from columns treated with 10^{-9}M GnRH (Fig. 17) and 10^{-8}M PMA (Fig. 18). Initial trials (n = 3 dispersions, using duplicate chambers for each dispersion) with the circular perifusion chamber (Chapter 2, Fig. 1) carried out at this time produced no clear evidence of pulsatile LH secretion. Two representative LH media profiles are shown. Treatment with 10^{-9}M GnRH produced three large LH peaks the first 24h in culture (Fig. 19), which did not coincide with the GnRH pulses administered. Cells stimulated with 10^{-8}M PMA (Fig.20) produced a single pulse after 2.5h in culture which again did not correspond to the PMA input.

These unresponsive perifusions coincided with a change in the batch of collagenase enzyme and a marked drop in cell viability from dispersions to around 30%.

3.2.2.2. Structural Observations

Histological sectioning of the cells and cytodex microcarriers taken from the viable perifusion columns, showed clumps of cells supported within a network of beads (Fig. 21). In contrast, the unresponsive columns possessed virtually no recognisable cell ultrastructure (Fig. 22). Nuclei undergoing karyorrhexis and vast numbers of collagen fibrils were observed. The granules present are typical of those containing prolactin (Chapter 2, Fig. 6). The lack of any surrounding membrane structure suggested that cells may have ruptured releasing the cytosolic contents.

Immunohistochemistry of the cell culture inserts using the immunogold localisation technique (**2.2.6.3.**) revealed isolated LHβ immunopositive cells with the majority of cell profiles being immunonegative (Fig. 23). Scanning electron micrographs of the same cell culture inserts revealed very few intact cells (Fig. 24), the majority of the area being covered with the remains of cellular outlines (Fig. 25). Live cells fixed on a cell culture insert immediately after dispersion are shown for structural comparison (Fig. 26). Transmission electron micrographs of these samples showed most cell profiles present to be agranular, the few granules visible being typical of those containing prolactin (Fig. 27).

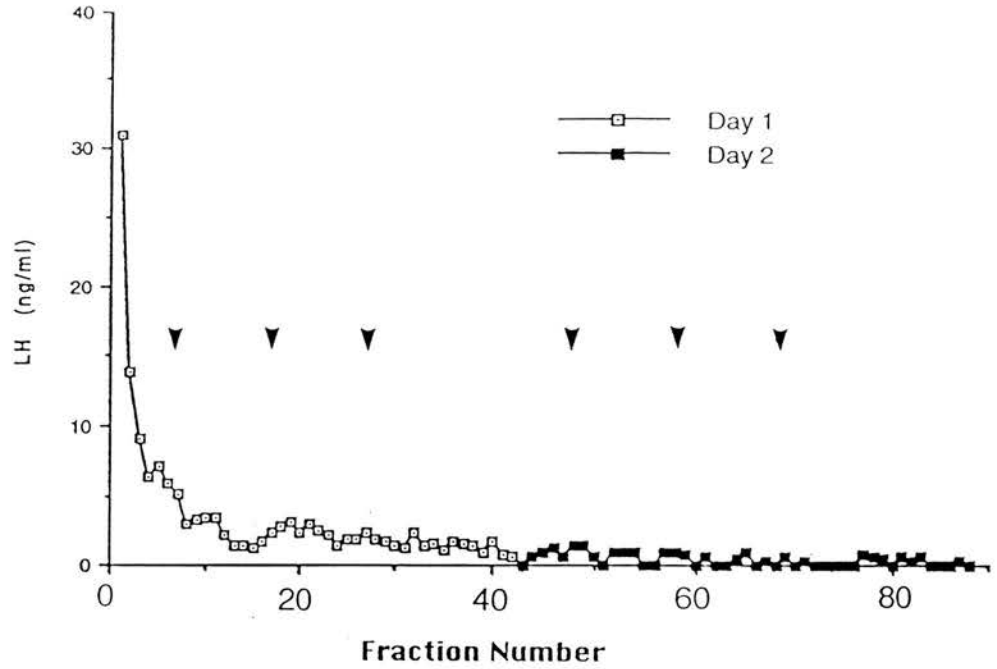


Figure 17. Representative pattern of LH secretion from dispersed pituitary cells using syringe columns, challenged with GnRH (10^{-9}M) over two days in perifusion cell culture. GnRH pulses (arrowheads) did not induce pulsatile LH release.

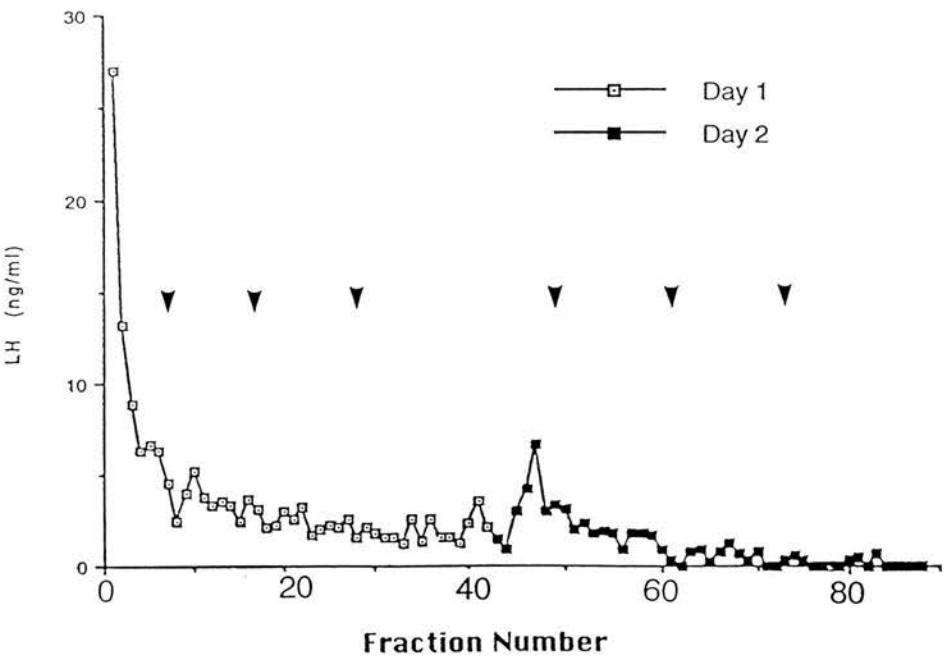


Figure 18. Representative pattern of LH secretion from dispersed pituitary cells using syringe columns, challenged with PMA (10^{-8}M) over two days in perifusion cell culture. PMA pulses (arrowheads) did not induce pulsatile LH release.

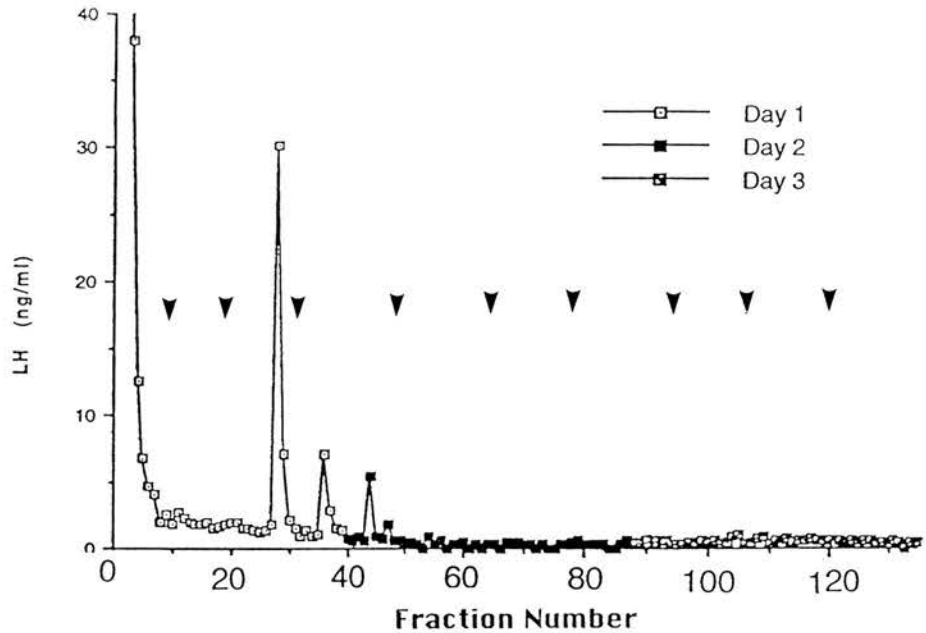


Figure 19. Representative pattern of LH secretion from dispersed pituitary cells using a circular perifusion chamber, challenged with GnRH (10^{-9}M) over three days in perifusion cell culture. The three LH pulses produced did not correspond to the times of the GnRH input (arrowheads).

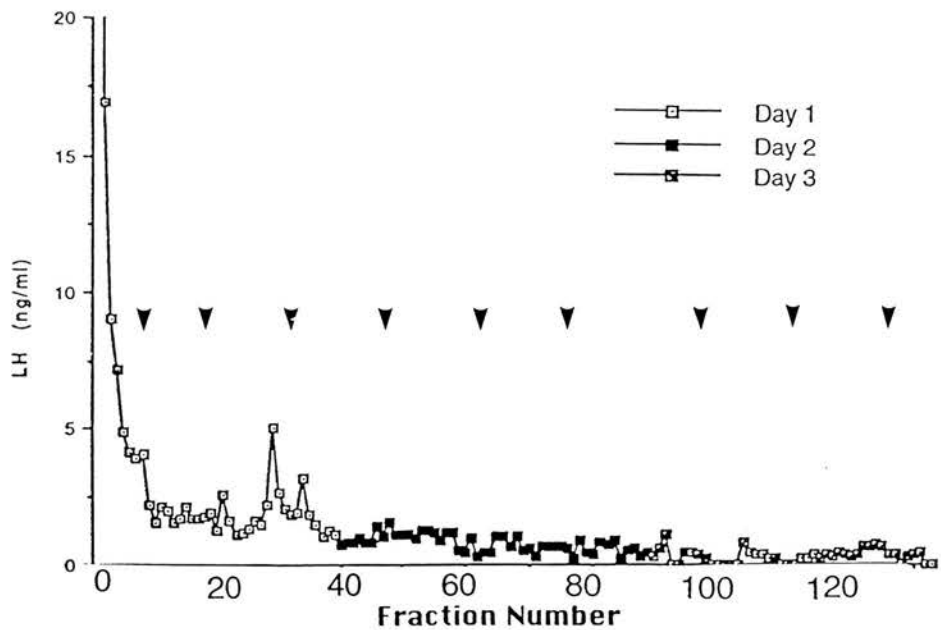


Figure 20. Representative pattern of LH secretion from dispersed pituitary cells using a circular perifusion chamber, challenged with PMA (10^{-8}M) over three days in perifusion cell culture. The elevations in basal LH secretion did not correspond to the times of the PMA input (arrowheads).

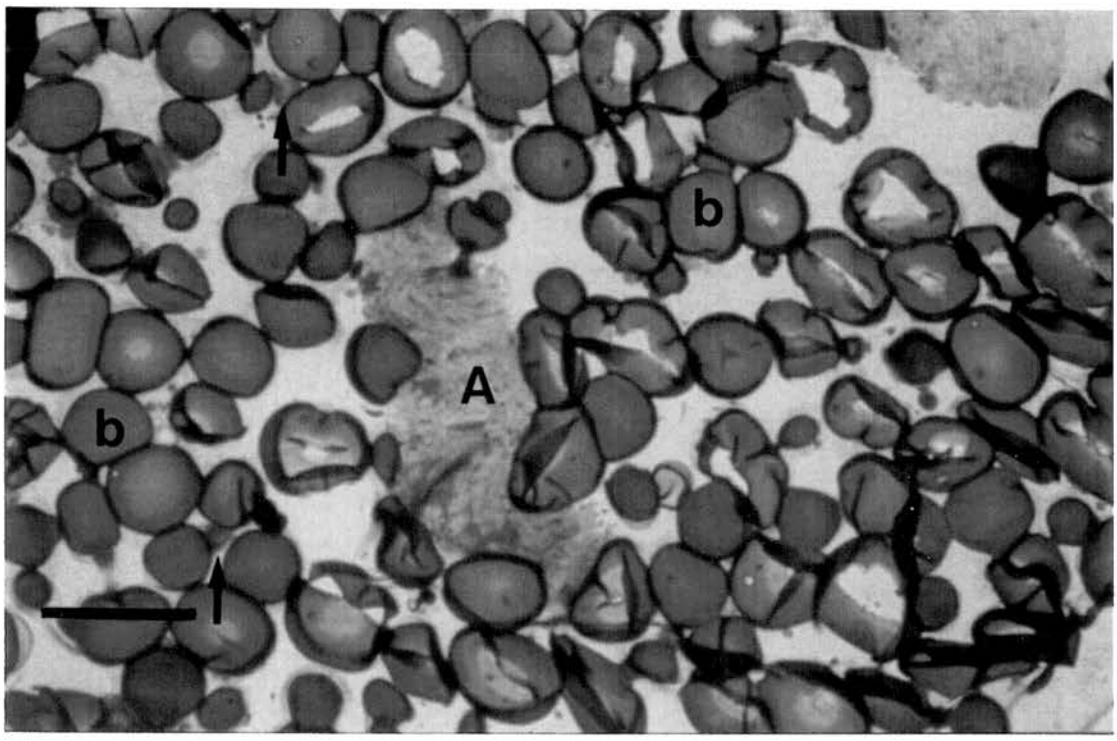


Figure 21. Histological micrograph, counterstained with 1% toluidine blue, showing dispersed pituitary cells within the perifusion column. Cells and connective tissue were found mostly in large aggregates (A) between a supporting matrix of cytodex beads (b). Occasionally, smaller tissue fragments were seen to attach to individual beads (↑). Magnification = 70X. Scale bar = 290μm.

Figure 22. Transmission electron micrograph of tissue cultured in the perifusion columns. No evidence of intact cell ultrastructure was found. Granules (g) structurally similar to those previously shown to contain prolactin (Fig. 5) or growth hormone (Thorpe *et al* 1990) were present throughout the tissue mass and were not enclosed by any visible cell membane. Collagen (c), with a banded periodicity typical of type IV, and nuclei (N) undergoing karyorrhexis, were abundant. The overall tissue morphology was characteristic of dead and dying cells. Magnification = 9800X. Scale bar = 2.1mm.

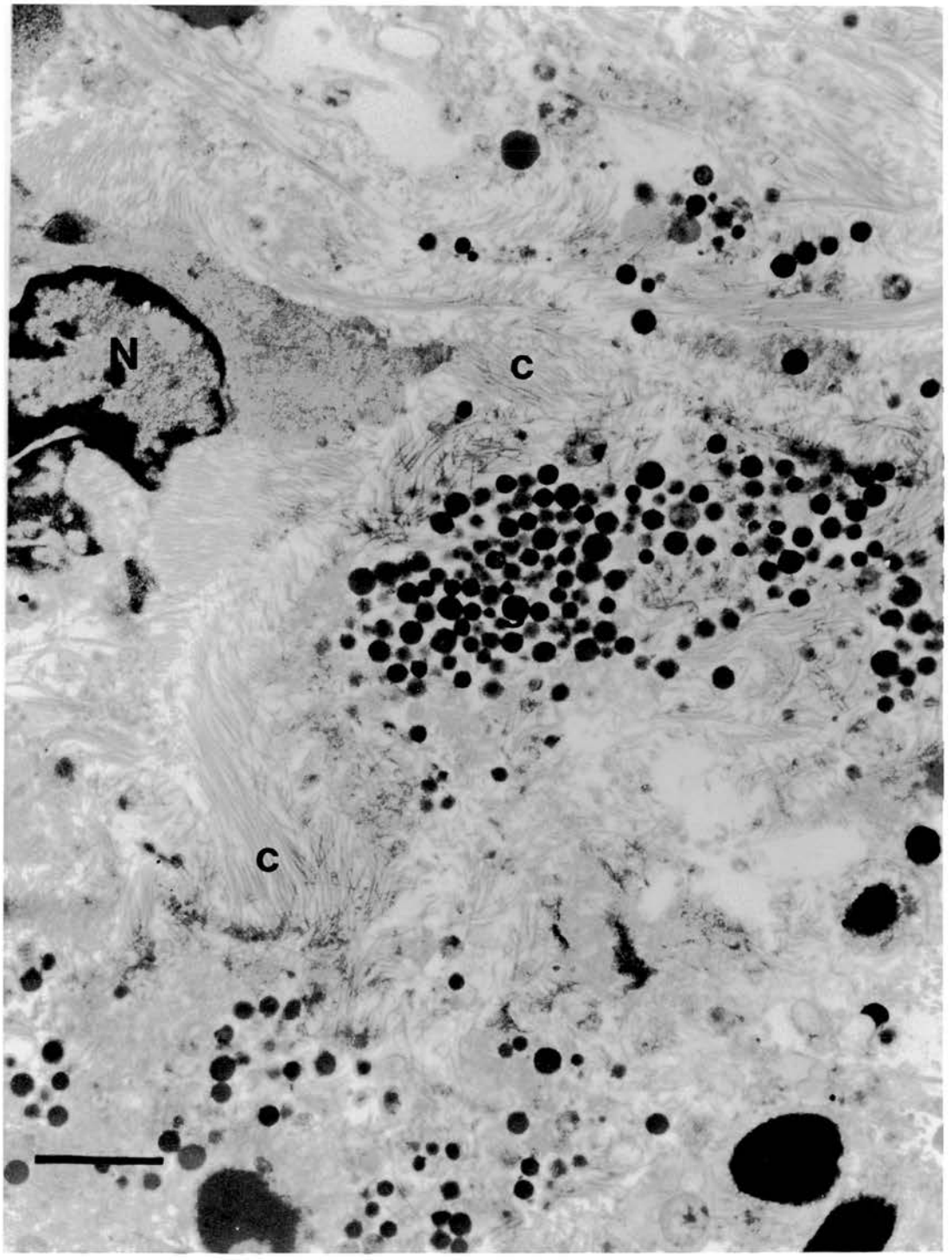
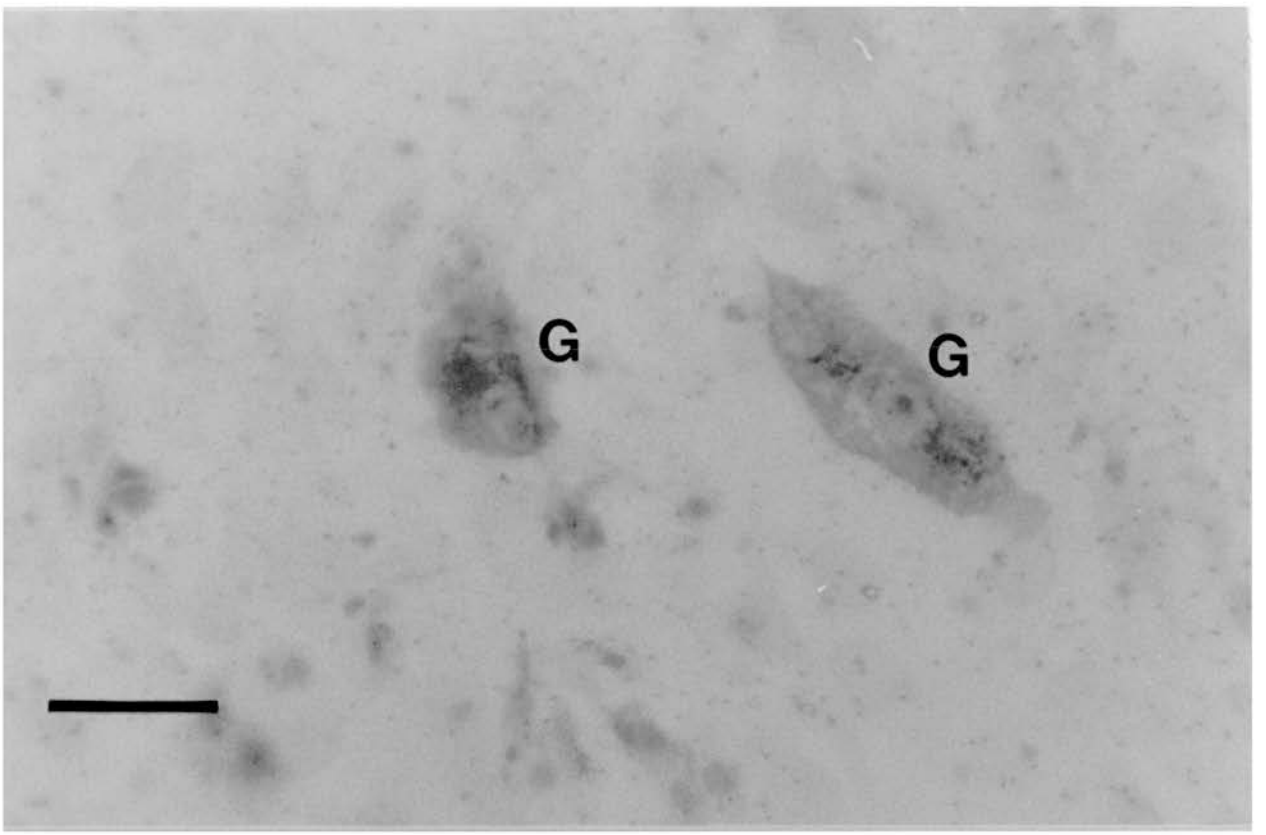

N
C
C

Figure 23. Histological micrograph showing the presence of LHβ subunit in dispersed gonadotrophs (G) stained using the immunogold method on a cell culture membrane. Gonadotrophs, with LHβ immunoreactivity clearly localised in the cytoplasm, were present in very small numbers, and displayed an elongated cell profile. Magnification = 400X. Scale bar = 50mm.

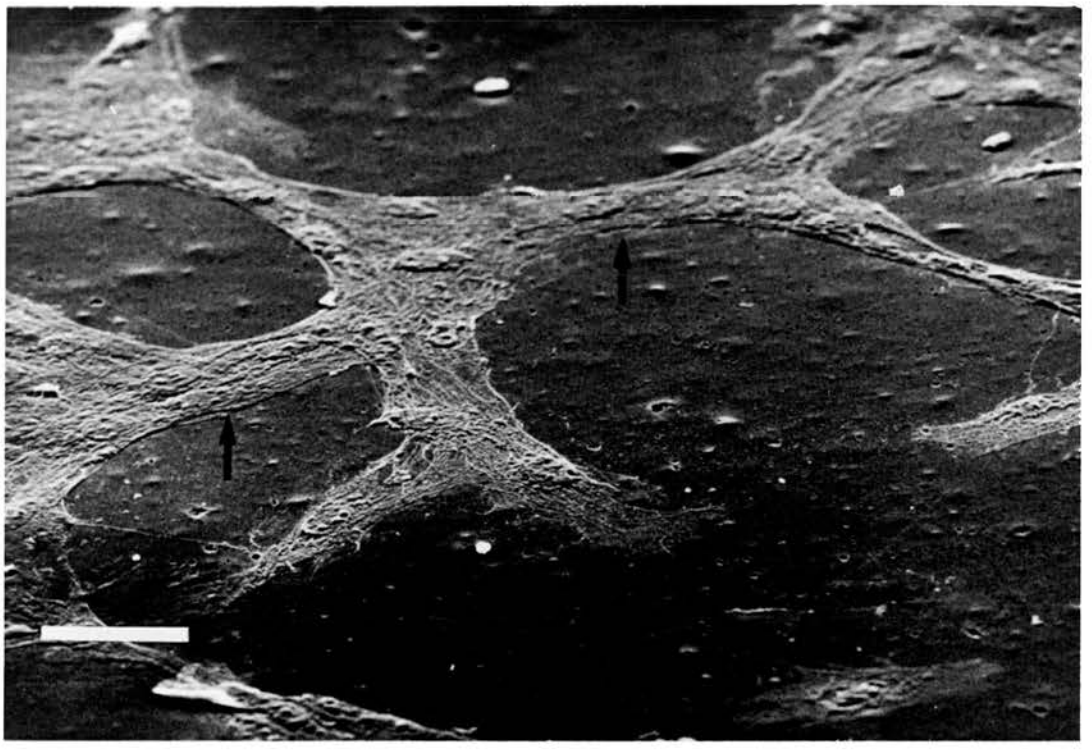
Figure 24. Scanning electron micrograph showing dispersed gonadotrophs attached to a cell culture membrane. Some cells (↟) were spherical with connective tissue remnants attached, whilst other were flattened (↟↟) and attached to the membrane. Fibroblasts (F), displaying a typical plaque-like morphology, were more abundant than the pituitary cells. Magnification = 1000X. Scale bar = 20mm.


G
G


E
P

Figure 25. Scanning electron micrograph showing the view typical of the majority of the cell culture membrane. No clearly identifiable cellular structures were present, connective tissue and necrotic cellular outlines (↟), possibly from fibroblasts, being all that remained. Magnification = 480X. Scale bar = 40mm.

Figure 26. Scanning electron micrograph illustrating cell morphology immediately after enzymatic dispersion. Intact pituitary cells (p) were broadly spherical and surrounded by large quantities of connective tissue (CT) and red blood cells (↟). Some pituitary cells appeared damaged (↟ ↟) whilst most of the red blood cells exhibited crenellation. Magnification = 1800X. Scale bar = 11mm.



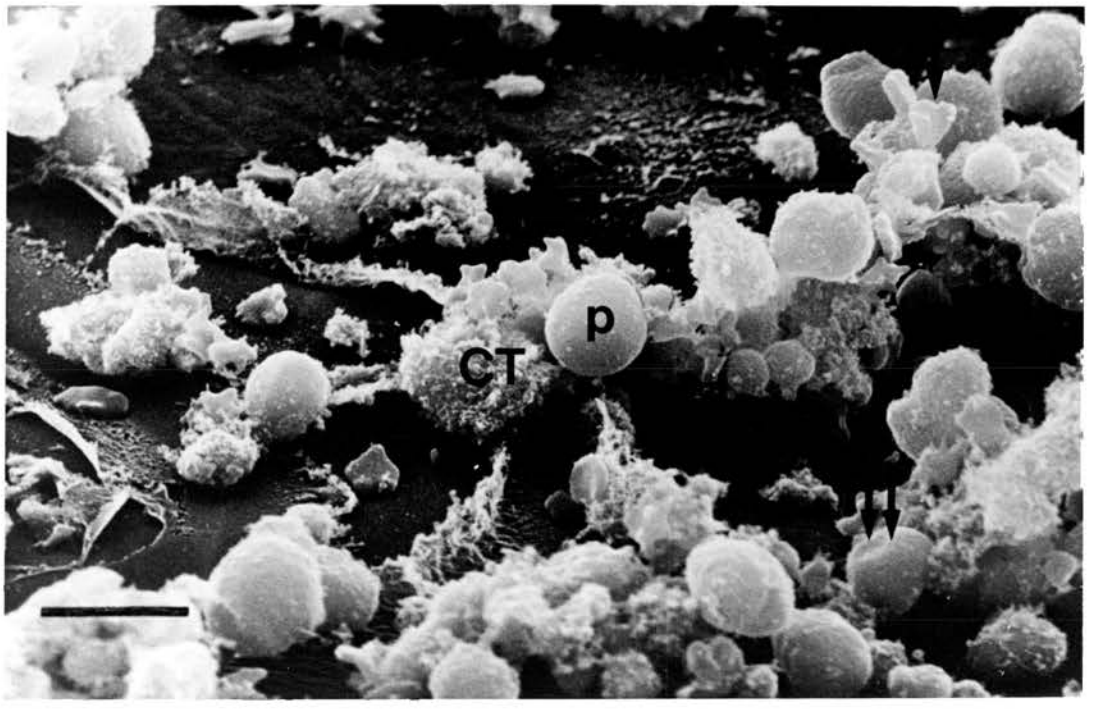
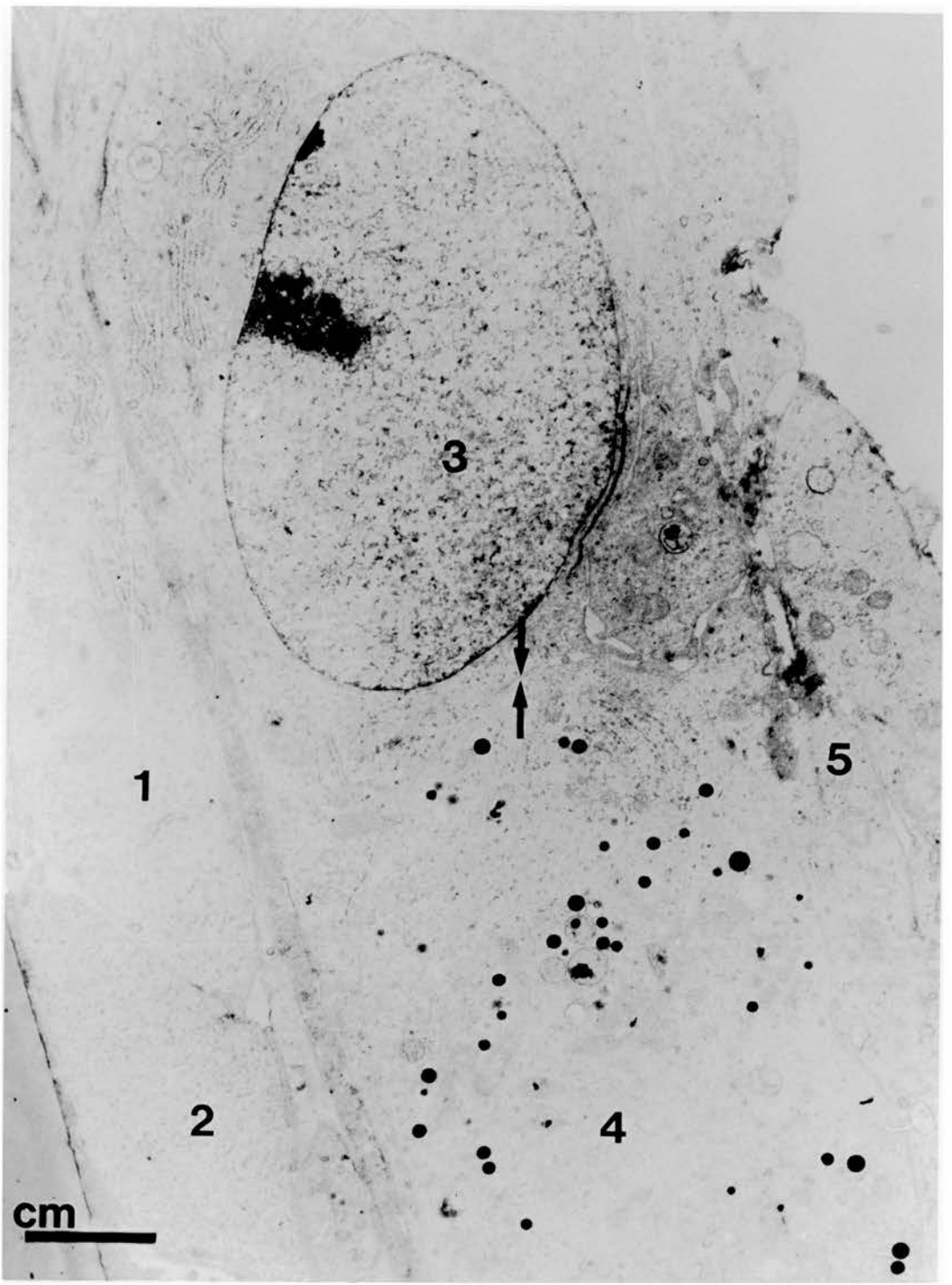

p
CT

Figure 27. Transmission electron micrograph showing the ultrastructure of cells attached to cell culture membranes. Although cells were distributed sparsely across the culture membranes (cm), occasionally layers of cells were observed. In this micrograph, at least five different cell profiles (1, 2, 3, 4 and 5) may be present. Most cell profiles were agranular with any granules present being structurally similar to those that contain prolactin (Fig. 5). Cell membranes were in close apposition (↟) throughout the aggregate. Magnification = 12500X. Scale bar = 1.6mm.


3
1
5
2
4
cm

3.2.3 Discussion

The perifusion experiments undertaken have attempted to duplicate the pulsatile GnRH input to which pituitary cells are exposed *in vivo*. After two isolated successful stimulations of a pulsatile LH response, the change of batch of the collagenase enzyme used appeared to be responsible for the loss of viability of the system.

The ability of the dispersed cells to remain in clusters may be of critical importance in the production of an LH pulse. Using rat pituitary cell aggregates, Van Der Schueren *et al* (1982) demonstrated pulsatile release of LH after 3 weeks in culture. The same authors noted that 'within a few days the aggregated pituitary cells became organized in a tissue-like configuration'. Furthermore, cells were joined by junctional complexes and in some instances, the intercellular space was filled with a basal lamina-like material. Prior to aggregation, trypsin was used to enzymatically disperse the cells. Previous studies with dispersed, isolated sheep pituitary cells in perifusion apparatus have shown a loss of sensitivity to GnRH stimulation after 24h (Dr. J. Brooks, unpublished observations). It may be that the intercellular associations within each aggregate allow the regeneration of the GnRH receptor which is thought to be removed during trypsinization (Dr. T.A. Bramley, personal communication).

The absence of any increased amplitude on the second day of PMA stimulation suggested that bypassing the membrane receptor to activate a protein kinase C directly leads to a generalised emptying of the intracellular LH stores which may continue until the stores are exhausted. The high pulse amplitudes observed on the first day and the subsequent decrease on the second, may be a reflection of this mechanism. Indeed the decreased response to the exocytotic stimulus with time is similar to the effect seen in the static culture experiments detailed above (**3.1.**). The greater LH response on day two of GnRH stimulation suggests that GnRH may promote either the movement of hormone or the recruitment of cells into the releasable pool (Smith and Neill 1987; Neill *et al* 1987). This may be similar to the GnRH priming effect on the release of LH first reported in the female rat (Aiyer *et al* 1974b). In the mouse, GnRH causes the migration of granules to the periphery of the gonadotroph associated with a greater release of LH in the second hour of GnRH stimulation as compared to the first (Lewis *et al* 1986). It is also possible that *de novo* synthesis of LH may contribute to the apparent increased responsiveness of the gonadotrophs during the second day in perifusion.

3.3. Attempts to Re-Establish Cell Viability and Responsiveness

The loss of viability of cells following enzymatic dispersion and the subsequent lack of responsiveness to GnRH and PMA coincided exactly with a change in the batch of collagenase used for dispersions. Extensive trials were therefore undertaken using differing collagenase dispersion methods in an attempt to produce viable cells which responded reproducibly to a secretory stimulus.

3.3.1. Materials, Methods and Results

Adult blackface and texel ewe heads were obtained from a local slaughter house. Pituitaries were excised and cells dispersed using the collagenase dispersion protocol previously described (**2.1.1.**). Different types of collagenase enzyme from a variety of sources over a number of incubation times were used. Enzyme sources, mixtures, concentrations, incubation times and cell viabilities together with LH production during a 2h challenge in static culture with 10^{-9}M GnRH are shown in Table 1.

Methods were repeated over three separate dispersions, with each treatment carried out using cells in three identical culture wells. No significant stimulation of LH release was achieved with any of the enzymes used.

Further studies were conducted on the effect of the length of GnRH stimulation using cells dispersed with Worthington collagenase (Fig. 28). No significant stimulation of LH release was achieved following application of 10^{-9}M GnRH for 30, 60 or 120mins.

3.3.2. Discussion

Despite extensive trials of different collagenase enzymes, it proved impossible to produce viable, responsive cells suitable for culture in the perifusion apparatus. The low viabilities obtained from the dispersions suggested that the enzymes were causing excessive cell damage. This phenomenon did not appear to be time dependent as no significant differences in viability were observed over 30, 45, 60 and 75 minute incubations with type IA collagenase (Sigma). The significant ($P<0.05$) increase in basal secretion shown in the 30min enzyme incubation with type IA collagenase over the 45, 60 and 75min enzyme incubations may be attributed to greater numbers of cells within the culture wells. Following this shorter incubation, mechanical trituration resulted in

Separation method			LH secretion over 2h incubation		
Enzymes	**Concentration**	**Incubation Time (mins at 37°C)**	**Cell Viability (%)**	**Basal**	**GnRH (10^{-9}M)**
Type IA Collagenase (Sigma)	0.1%	30	34.6 ± 6.1	513.0 ± 76.3*	447.8 ± 25.7
		45	43.5 ± 11.3	260.0 ± 29.5	231.9 ± 3.3
Hyaluronidase (Sigma)	0.1%	60	48.7 ± 15.1	331.2 ± 38.8	344.0 ± 47.6
		75	30.1 ± 4.9	316.6 ± 7.1	314.4 ± 10.2
Collagenase Blends (Sigma): F	0.1%	60	0	-	-
L	0.1%	60	31.6 ± 3.8	248.9 ± 121.7	416.6 ± 161.2
N	0.1%	60	13.2 ± 7.1	115.6 ± 32.9	137.8 ± 32.9
H	0.1%	60	41.2 ± 7.5	185.0 ± 63.8	246.1 ± 32.7
Worthington Crude Collagenase (Cambridge Biosciences)	0.1%	60	63.2 ± 24.7	43.1 ± 3.8	52.7 ± 9.7
		48h at 4°C	29.2 ± 6.1	109.6 ± 12.8	122.8 ± 19.6
Worthington Crude Collagenase Trypsin Inhibitor (Sigma) DNase (Sigma)	0.1% 1% 0.01%	60	21.4 ± 7.9	61.4 ± 8.9	74.4 ± 10.2

Table 1. Variations of the collagenase dispersion method used in an attempt to produce gonadotrophs which would respond reproducibly to GnRH. Cells were cultured for 48h at 37°C. Medium was removed and replaced with medium alone (basal) or medium containing GnRH (10^{-9}M). The release of LH was measured over the following 2h. Results are mean ± SEM of triplicate replicates from three separate dispersions. There was no significant difference between LH released from basal and GnRH treatment wells.

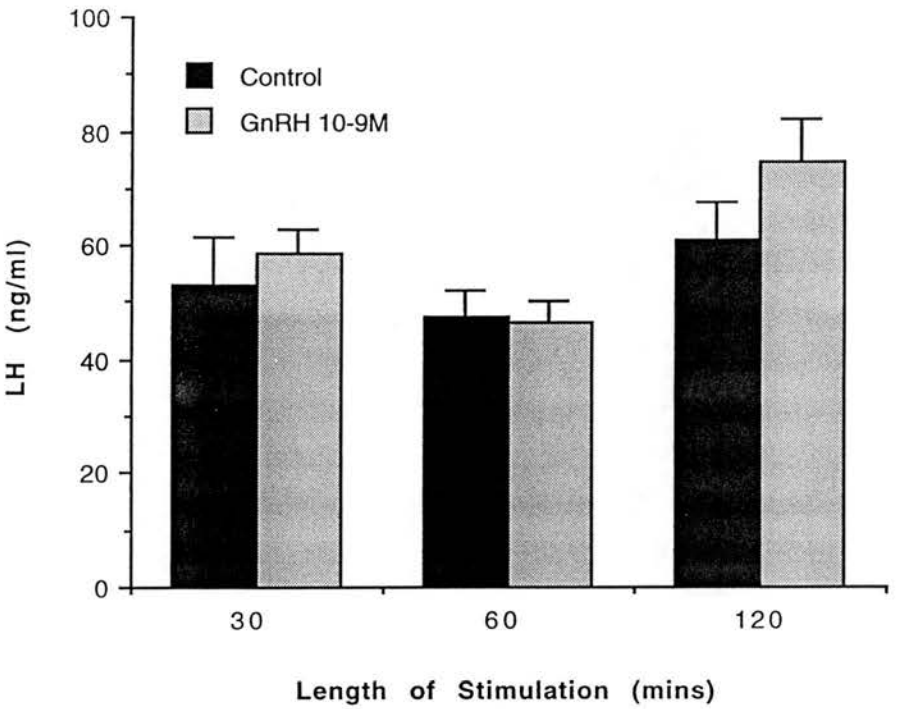


Figure 28. Effect of length of 10^{-9}M GnRH stimulation on LH secretion from cells 48h after dispersion using Worthington crude collagenase. Medium was removed and replaced with medium alone (basal) or medium containing GnRH (10^{-9}M). The release of LH was measured over 30, 60 and 120 minutes. Results are mean ± SEM of triplicate replicates from three separate dispersions. There was no significant difference between LH released from control and GnRH treatment wells.

large cell fragments which may have led to errors in estimated cell concentrations using the haemocytometer.

The development of a dynamic culture system which allows the pulsatile administration of releasing hormones and the constant removal of cell waste products should provide an accurate model for the *in vivo* situation. However, a lack of correct cell-cell association and surrounding connective tissues may affect both the ease of hormone release and the mechanism by which it is achieved. Detailed ultrastructural *in vivo* investigations of subcellular events associated with LH release in the sheep are a prerequisite to allow the duplication of physiological events *in vitro*.

CHAPTER 4

SYNTHESIS AND SECRETION OF LH DURING THE FOLLICULAR PHASE

Introduction

During the follicular phase, waves of follicles develop in the ovary. and one of these is selected as the dominant follicle which ovulates in response to the preovulatory surge of LH released from the pituitary. The increased LH pulse frequency of more than 1 pulse/h in the follicular phase (Baird 1978) is a result of an increase in GnRH pulse frequency (Clarke *et al* 1987) with a concomitant decrease in GnRH pulse amplitude, both effects being mediated by oestradiol (Evans *et al* 1994). Greater pituitary responsiveness to GnRH occurs via an increase in GnRH receptor numbers (Karsch 1987). The increased GnRH receptor number is a reflection of increased GnRH receptor mRNA levels and leads to elevated pituitary GnRH binding capacity (Brooks *et al* 1993).

It is well established that *in vivo* GnRH, on primary application, can alter the responsiveness of the gonadotroph allowing subsequent challenges to induce enhanced LH release: this is known as GnRH priming (Aiyer *et al* 1974b; Fink *et al* 1976; Pickering and Fink 1976, 1979; Crighton and Foster 1976, 1977; Stelmasiak and Galloway 1977). The mechanism involves protein kinase C, which initiates the release of arachadonic acid via the upregulation of phospholipase A_2 activity (Thomson *et al* 1994). Gonadotrophin surge attenuating factor, present in steroid-free human follicular fluid, has been shown to reduce the GnRH priming effect in ovine pituitary perifusion studies (Fowler *et al* 1993).

In cultured rat pituitary cells, GnRH treatment stimulates the movement of secretory granules to peripheral regions of the cell near the plasma membrane (Tixier-Vidal *et al* 1975). The treatment of mouse pituitaries *in vitro* with GnRH induces a priming effect in gonadotrophs such that secretory granules are translocated to subplasmalemmal regions around the entire cell circumference, thus allowing a significantly greater release of LH in the second hour of exposure (Lewis *et al* 1986). It has not been determined if this phenomenon occurs in other species.

The experiment was designed to: 1. establish if the ultrastructural changes in rat and mouse gonadotrophs accompanying GnRH priming *in vitro* were present *in vivo* in the

sheep related to the stage of cycle and 2. relate any such changes to the regulation of LHβ gene expression throughout the ovine oestrous cycle.

4.1 Materials and Methods

4.1.1 Animals and Experimental Design

Thirty Welsh Mountain ewes were studied during the breeding season in February 1993. Ewes were 3-4 years old and weighed 30-45kg. Animals were synchronised initially by withdrawing progestagen sponges. Animals were then allocated randomly to 6 groups (n=5 per group) and one group killed in luteal phase. On the morning of the experiment, one jugular vein was cannulated and an intramuscular injection of cloprostenol, a synthetic analogue of prostaglandin $F_{2\alpha}$ (PG), given to induce luteolysis. After a further 16h, the onset of behavioural oestrus was detected by introducing a vasectomized ram at 2 hourly intervals, individually with each animal. Five ewes were killed at each of the following times: follicular phase (18h after PG); oestrus onset; oestrus onset plus 9h; oestrus onset plus 24h. Of the animals which were tested for oestrus, 5 failed to exhibit behavioural oestrus and were therefore injected with 50mg of the GnRH agonist buserelin (Hoechst, Frankfurt, Germany), to induce an LH surge, and killed 1hr later. These times were chosen to provide pituitary tissue at points throughout the oestrous cycle likely to demonstrate the greatest intracellular changes namely before, during and after the preovulatory LH surge. At death, pituitaries were dissected out and processed within 5mins of death.

4.1.2. Tissue Preparation and Immunocytochemistry

A 1mm thick transverse section taken through the mid-plane of the adenohypophysis was processed for ultrastructural immunocytochemistry as previously described (**2.2.6.2.**). Gonadotrophs were immunoidentified by the localisation of LHβ subunit using NIDDK anti ovine LH antisera at a dilution of 1:800. The dilution was chosen by determining the highest dilution at which gold particles were absent from nuclear profiles. Antibody specificity was confirmed by replacing the primary antibody with normal serum and preabsorption with purified ovine LH.

4.1.3. Measurement of mRNA Abundance

Following the removal of a 1mm tissue slice for immunocytochemistry, the remainder of the adenohypophysis was frozen in liquid nitrogen. Total RNA was extracted and LHβ mRNA abundance determined by Northern analysis (**2.2.7.**) using a cDNA probe. Results were quantified using a Phosphor Imager (Molecular Dynamics).

4.1.4. Quantitation of Changes in Granule Localisation

The percentage of polarised cells (see **4.2.**) throughout the oestrous cycle was determined by counting 20 cells per animal, located using systematic random sampling (**2.2.6.4.**). Cells were deemed to have polarised when approximately half of the cytoplasm was devoid of granules.

4.1.5. Stereology

To determine the relationship between the sizes of granules present and the possible secretory status of the cell, gonadotrophs were selected based on the extent to which the granule distribution was polarised within the cytoplasm (**4.2.2.**). Individual animals were analysed at each time point to allow any changes in granule size distribution to be related to exact plasma LH concentrations. Non-polarised immunoidentified gonadotrophs were randomly selected at day 12 luteal phase. Polarised cells in which approximately 50% of the cytoplasm was devoid of secretory granules were sampled just prior to the preovulatory LH surge whilst polarised cells with approximately 75% agranular cytoplasm were sampled in mid-LH surge. Gonadotrophs in which only a small number of granules remained in the periphery of the cell nearest the blood vessel were selected at the peak of the LH surge. Cells were located using a systematic random sampling technique with a minimum of 1000 profile diameters, from between 13-20 cells, being measured per animal. Electron micrographs of each cell, taken at magnifications of 4600 and 7700X were analysed by measuring granule profile diameters and calculating the distribution of granule sizes using the Schwartz-Saltykov diameter analysis (**2.2.6.4.**).

4.1.6. Blood Sampling Schedule

Blood samples (3ml) were taken hourly throughout the study. In addition, blood samples (2.5ml) were collected every 10 mins for 4 hours, 14-18h after PG in the luteal

and follicular control groups and 24-28h after PG in the oestrus, oestrus plus 9h, oestrus plus 24h and GnRH agonist induced LH surge groups, to measure the pulsatile secretion of LH.

4.1.7. Plasma Radioimmunoassay

Plasma concentrations of LH were measured in duplicate using the radioimmunoassay described in Chapter **2.2.3.1.** (assay sensitivity = 0.3ng/ml, intra-assay coefficient of variation= 10.9%). Oestradiol concentrations were measured in terminal samples from oestrus, oestrus plus 9h, oestrus plus 24h and buserelin induced LH surge groups using the method previously described (**2.2.3.2.**) (assay sensitivity = 0.2pg/ml, intra-assay coefficient of variation = 12.9%).

4.1.8. Measurement of GnRH Binding and Pituitary Content of LH

Pituitary glands, previously frozen in liquid nitrogen after removal from the animal, were immersed in cold 0.3M sucrose/1mM EDTA/tris HCl, pH 7.4 medium (SET) using 1ml of medium/ 100mg tissue. The preparation was homogenised for 30secs on ice using a Polytron homogeniser (Kinematica, Lucerne, Switzerland). The pituitary homogenate was then analysed for LH content (assay sensitivity = 2ng/ml, intra-assay coefficient of variation = 11.6%) and GnRH-binding activity as previously described (**2.2.3.**).

4.1.9. Statistical Analysis

The levels of LHβ mRNA were analysed using one way analysis of variance followed by Fishers protected least significant difference (PLSD) test where a significant ($P<0.05$) interaction was found. The parameters of LH pulse secretion were determined using the Munro pulse analysis programme (Zaristow Software, Haddington, U.K.).

4.2. Results

4.2.1. Plasma Hormone Concentrations

The LH plasma profiles (Fig. 29) confirmed the correct timing of the collection of pituitaries. LH levels fell after catheterisation to give typical luteal phase values at the time of death. LH then rose slightly during the early follicular phase before returning

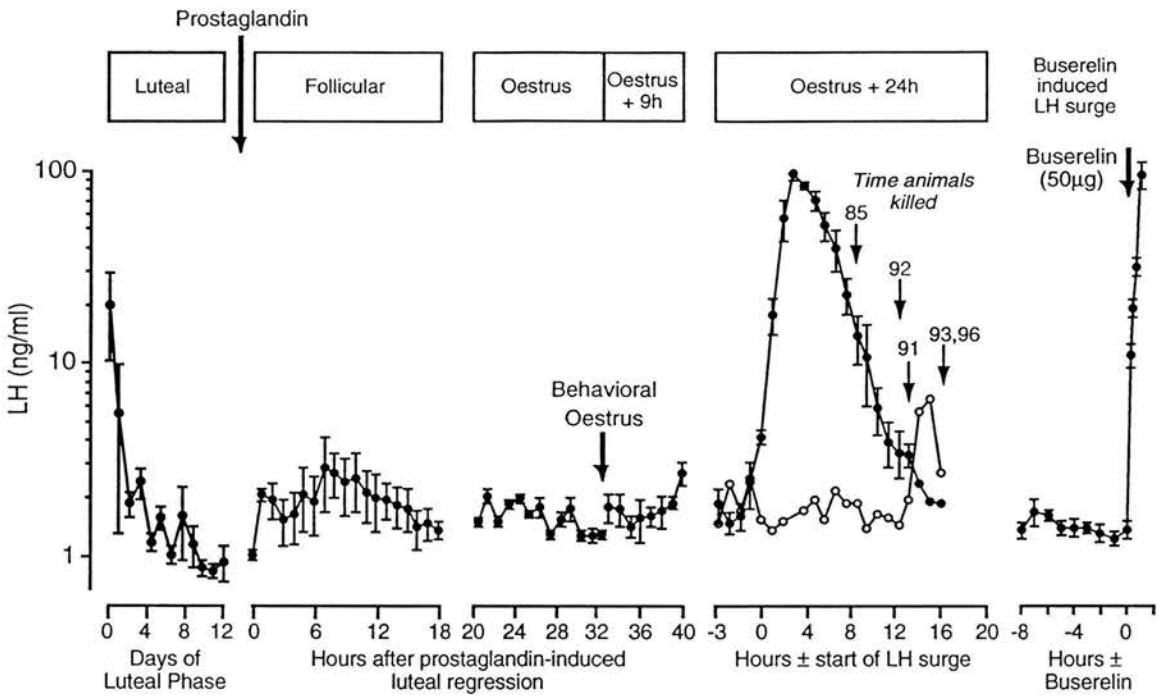


Figure 29. Changes in plasma concentrations of LH (mean ± SEM) from luteal through follicular phase. Daily LH concentrations are shown during the luteal phase with hourly samples taken throughout the follicular phase after prostaglandin-induced luteolysis. In the oestrus + 24h group results were aligned from the onset of the LH surge, defined as a sustained increase in plasma LH > 5ng/ml.

to low levels which were maintained throughout behavioural oestrus. Behavioural oestrus occurred at 33.6±1.0h after PG. At oestrus plus 9h the preovulatory LH surge had not been initiated, whilst at oestrus plus 24h, 4 out of 5 animals had produced a surge.

Parameters of pulsatile LH secretion are shown in Table 2.

Table 2. LH pulse frequency, pulse amplitude and basal concentrations 14-18h after PG in the luteal and follicular control groups and 24-28h after PG in the remaining follicular phase groups. Values are given as mean ± SEM and n = 5 in all groups. Pulse frequencies are expressed as pulses/6h to allow direct comparison between these and pulse frequencies in Chapters 5 and 6.

Animal Group	Pulse Frequency (Peaks/6h)	Pulse Amplitude (ng/ml)	Basal LH (ng/ml)
Luteal	1.2 ± 0.81	1.04 ± 0.20	0.69 ± 0.11
Follicular	0.8 ± 0.48	1.50 ± 0.48	1.32 ± 0.16
Oestrus	5.1 ± 1.62	1.04 ± 0.38	1.10 ± 0.17
Oestrus + 9h	6.6 ± 0.36	1.28 ± 0.37	1.05 ± 0.11
Oestrus + 24h	3.6 ± 1.40	0.98 ± 0.15	0.98 ± 0.15
Buserelin-Induced LH Surge	5.7 ± 1.53	1.22 ± 0.12	1.18 ± 0.42

Representative LH pulse profiles from individual ewes from each group are shown in Figure 30. Data confirmed that all ewes in each treatment group produced LH pulses.

Oestradiol concentrations in terminal plasma samples from oestrus, oestrus plus 9h and buserelin-induced LH surge groups showed no significant differences (Fig. 31). At oestrus plus 24h, after the LH surge, plasma oestradiol concentration decreased significantly ($P<0.05$). One animal in the oestrus plus 24h group which failed to produce a LH surge had an elevated plasma oestradiol concentration compared to members of the same group.

4.2.2. GnRH Binding and Pituitary LH Content

GnRH-binding activity showed no significant differences during the periods of luteal and follicular phase studied (Fig. 32). The induction of an LH surge with buserelin resulted in a non-significant decrease in GnRH-binding activity. In one animal from the oestrus plus 24h group which failed to produce an LH surge, GnRH binding activity was higher than for the remainder of the oestrus plus 24h group.

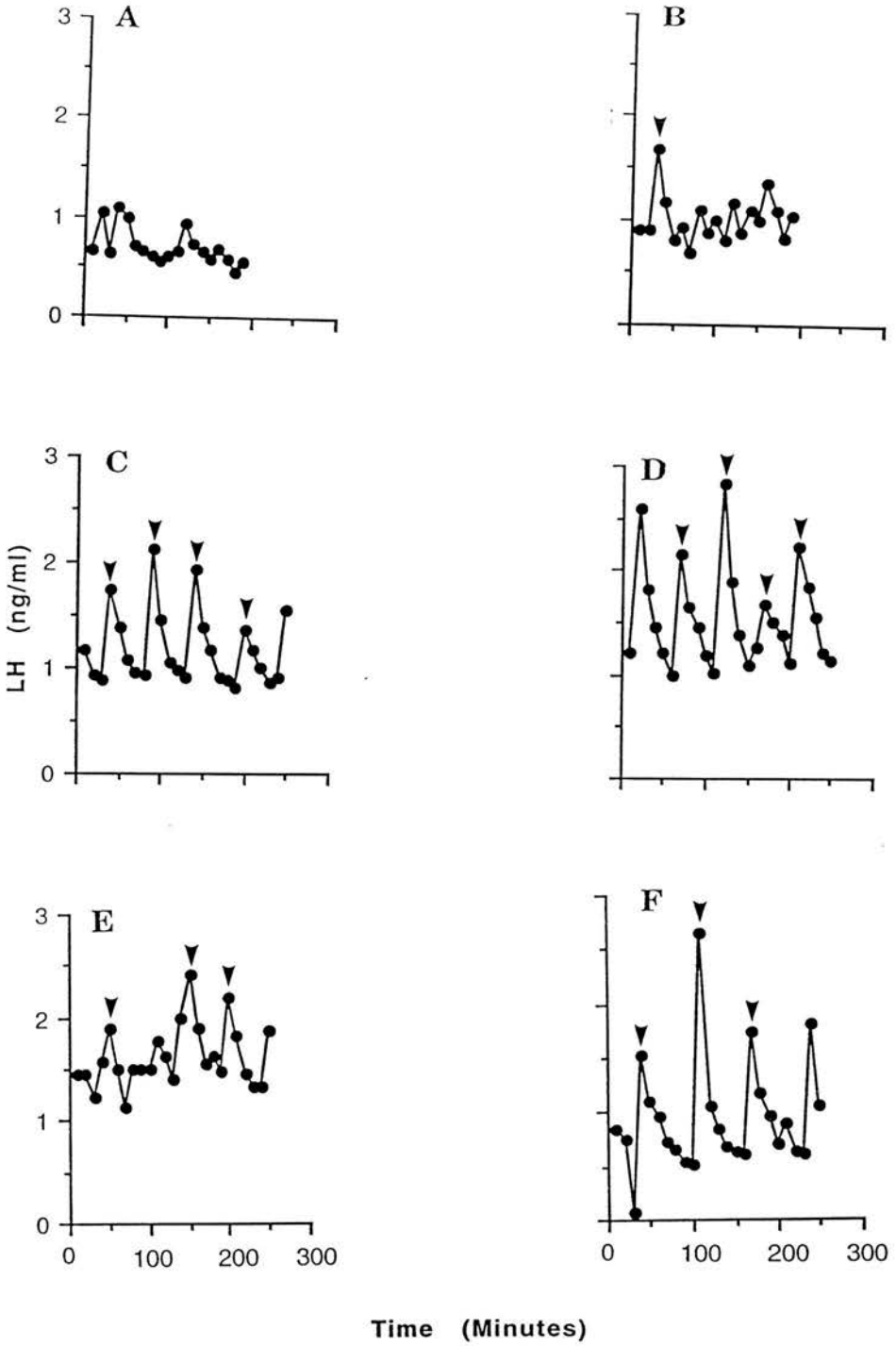


Figure 30. Representative profiles from individual ewes from day 12 luteal phase (A), early follicular phase 18h after prostaglandin-induced luteolysis (B), oestrus (C), oestrus plus 9h, before the LH surge (D), oestrus plus 24h, after the LH surge (E) and a buserelin-induced LH surge (F) showing the pulsatile pattern of LH secretion. Plasma samples were taken at 10 minute intervals for 3h on day 12 of the luteal phase and in early follicular phase, from 15-18h after prostaglandin, immediately prior to death. In the remaining groups, 10 minute samples were collected for 4h from 20-24h after prostaglandin-induced luteolysis. Arrowheads indicate significant LH pulses.

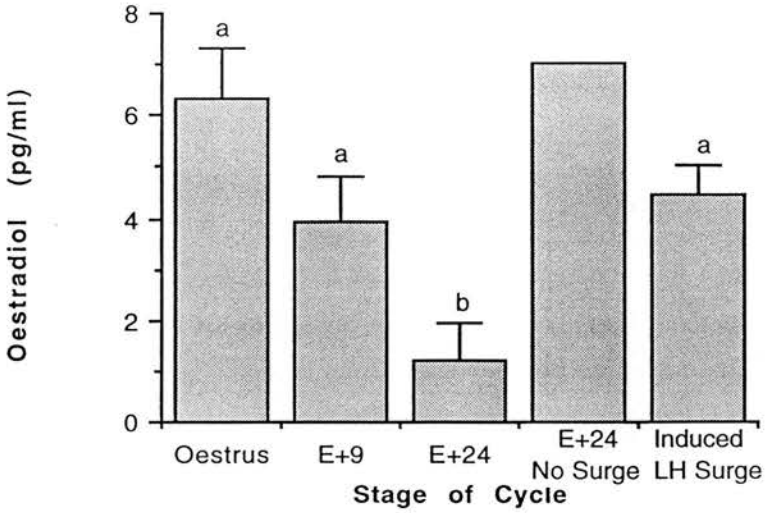


Figure 31. Terminal plasma concentrations of oestradiol from oestrus, oestrus plus 9h (before the LH surge), oestrus plus 24h (after the LH surge) and buserelin-induced LH surge groups. Values are given as mean ± SEM, n = 5 in all groups except oestrus plus 24h in which n = 4. One ewe in the oestrus plus 24h group failed to produce a LH surge and is shown separately. Data was analysed by one way ANOVA and different letters indicate significant ($P<0.05$) differences.

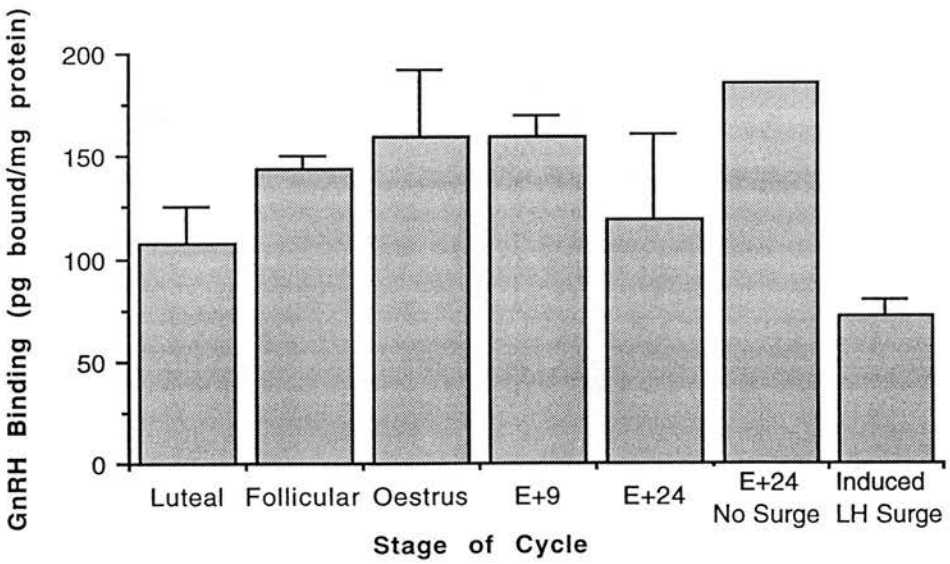


Figure 32. Changes in GnRH binding from luteal through the follicular phase. Values are given as mean ± SEM, n = 5 in all groups except oestrus plus 24h in which n = 4. One ewe in the oestrus plus 24h group failed to produce a LH surge and is shown separately. There were no significant differences between groups (one-way ANOVA).

Pituitary LH content showed a significant ($P<0.05$) decrease from early follicular phase (PG+18h) to oestrus (Fig.33). Concentrations remained suppressed until after the preovulatory LH surge (oestrus plus 24h). In the one animal from the oestrus plus 24h group which failed to produce an LH surge, LH content was increased, compared to the rest of the group, to a concentration around that measured in early follicular phase.

4.2.3. Analysis of LHβ mRNA Abundance

Analysis of LHβ mRNA abundance (Fig. 34) showed a significant ($P<0.05$) decrease at oestrus, before the LH surge. Levels appeared to rise at oestrus+9h before falling to their lowest value during the LH surge, although this change was not significant. A slight, non-significant, rise was evident at oestrus plus 24h.

4.2.4. Ultrastructural Observations

Post-embedding immunogold staining of ultrathin sections viewed under the transmission electron microscope showed the LHβ subunit located over electron dense granules and light dense bodies, as indicated by the position of 15nm gold particles (Fig. 35). A small proportion of granule profiles were LHβ immunonegative (see section **4.5.**). No evidence for either the existence of granule fusion figures or secretory granules of variable electron density (Tougard and Tixier-Vidal 1988) was seen. The larger electron dense granules in the adjacent cell were structurally typical of prolactin granules (Chapter 2, Fig. 5). A small proportion of cells with a similar granule structure were prolactin immunonegative. In the sheep, these cells contain growth hormone (Thorpe *et al* 1990).

In luteal and early follicular phase, 18h after PG induced luteolysis, 80% of gonadotrophs contained secretory granules that were distributed throughout the cytoplasm with no obvious bias towards the adjacent capillary (Fig. 36). This type of granule distribution was termed non-polarised. In the remaining 20% of gonadotrophs at these two stages of the cycle, the vast majority of granules had been translocated to the aspect of the cell juxtaposed to the nearest sinusoid. This distribution of granules within the cytoplasm, leading to the appearance of a polarised cell occurred, without exception, in the direction of the vascular system. At behavioural oestrus, around 45% of gonadotrophs displayed a polarised granule distribution with the remaining 55% non-polarised. By oestrus plus 9h (Fig. 37), just before the preovulatory LH surge, polarised gonadotrophs had become the more prevalent form, composing 75% of the total

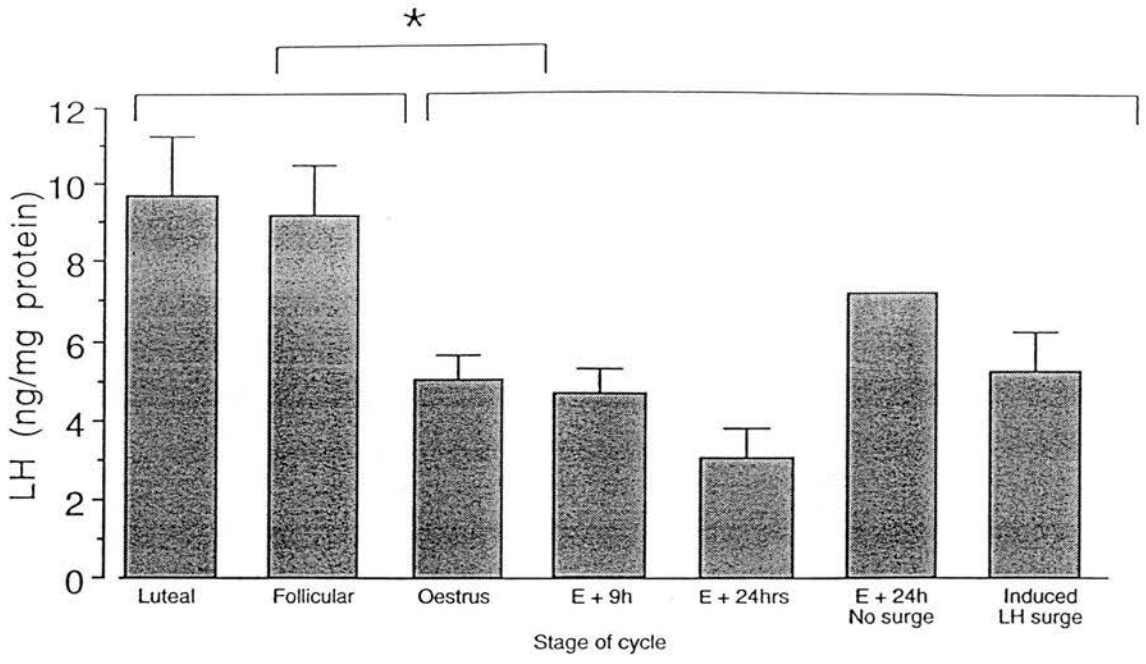


Figure 33. Changes in the content of LH in pituitaries collected from ewes in the luteal phase and at different stages throughout the follicular phase. Values are given as mean ± SEM, n=5 in all groups except oestrus plus 24h in which n=4. One ewe in the oestrus plus 24h group failed to produce an LH surge. Data was analysed by one-way ANOVA. * indicates a significant ($P<0.05$) difference.

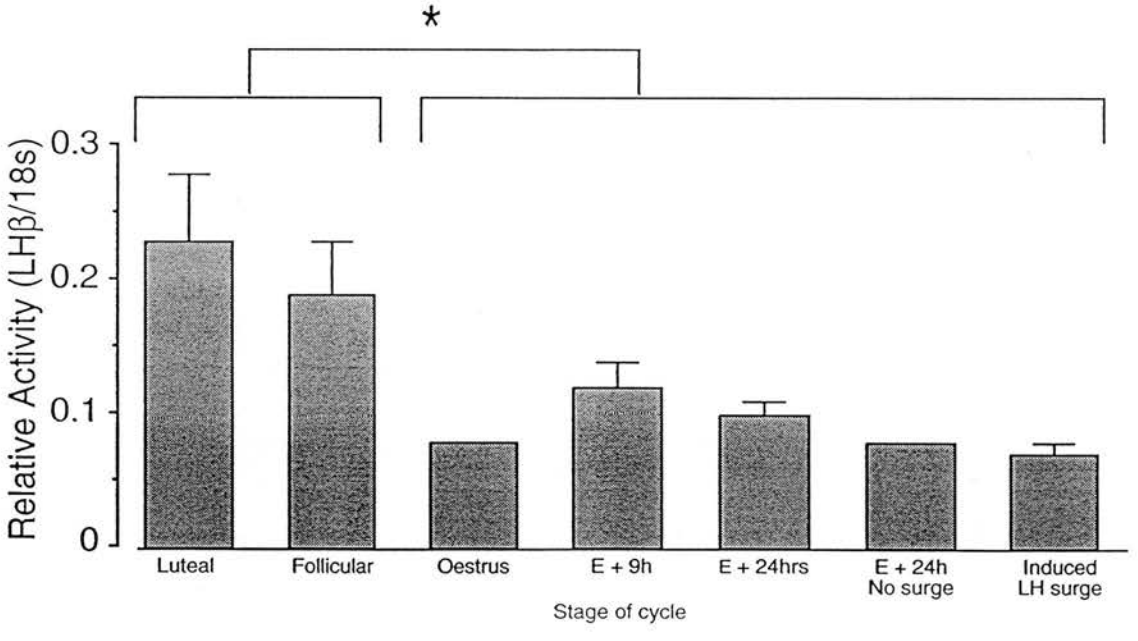


Figure 34. Changes in LHβ mRNA abundance from luteal through the follicular phase of the cycle, expressed relative to 18S ribosomal mRNA subunit. Blots were exposed for 2h and the results quantified using a Phosphor Imager. Values are given as mean ± SEM, n=5 in all groups except oestrus plus 24h in which n=4. One ewe in the oestrus plus 24h group failed to produce an LH surge. Data was analysed by one-way ANOVA. * indicates a significant ($P<0.05$) difference.

Figure 35. Transmission electron micrograph demonstrating the localisation of LHβ within a gonadotroph using immunogold labelling. The LHβ subunit, as identified by the presence of 15nm gold particles, was localised over electron dense secretory granules (↑) and light dense bodies (↑ ↑) throughout the cytoplasm. A number of granule profiles were LHβ immunonegative (↗). Specific staining was also absent from mitochondria (M) and the cell nucleus (N). Magnification = 40500X. Scale bar = 495nm.

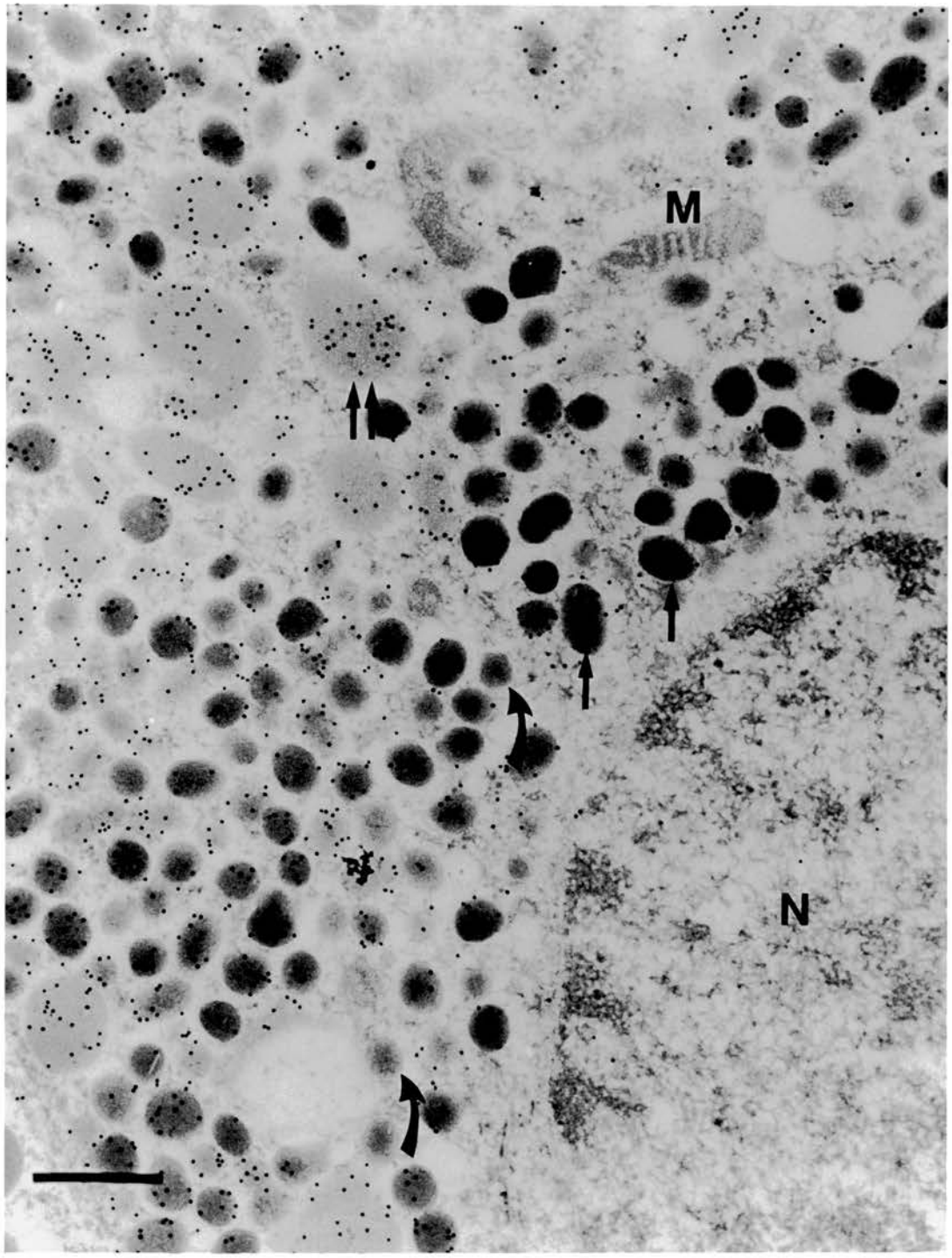

M
N

Figure 36. Transmission electron micrograph illustrating a gonadotroph (G), identified by the immunolocalisation of LHβ, during the luteal phase of the oestrous cycle. The secretory granules were distributed throughout the cytoplasm with no obvious bias towards the adjacent capillary (C). The surrounding cells contained granules structurally typical of those found in lactotrophs and somatotrophs. Magnification = 14000X. Scale bar = 1.4μm.

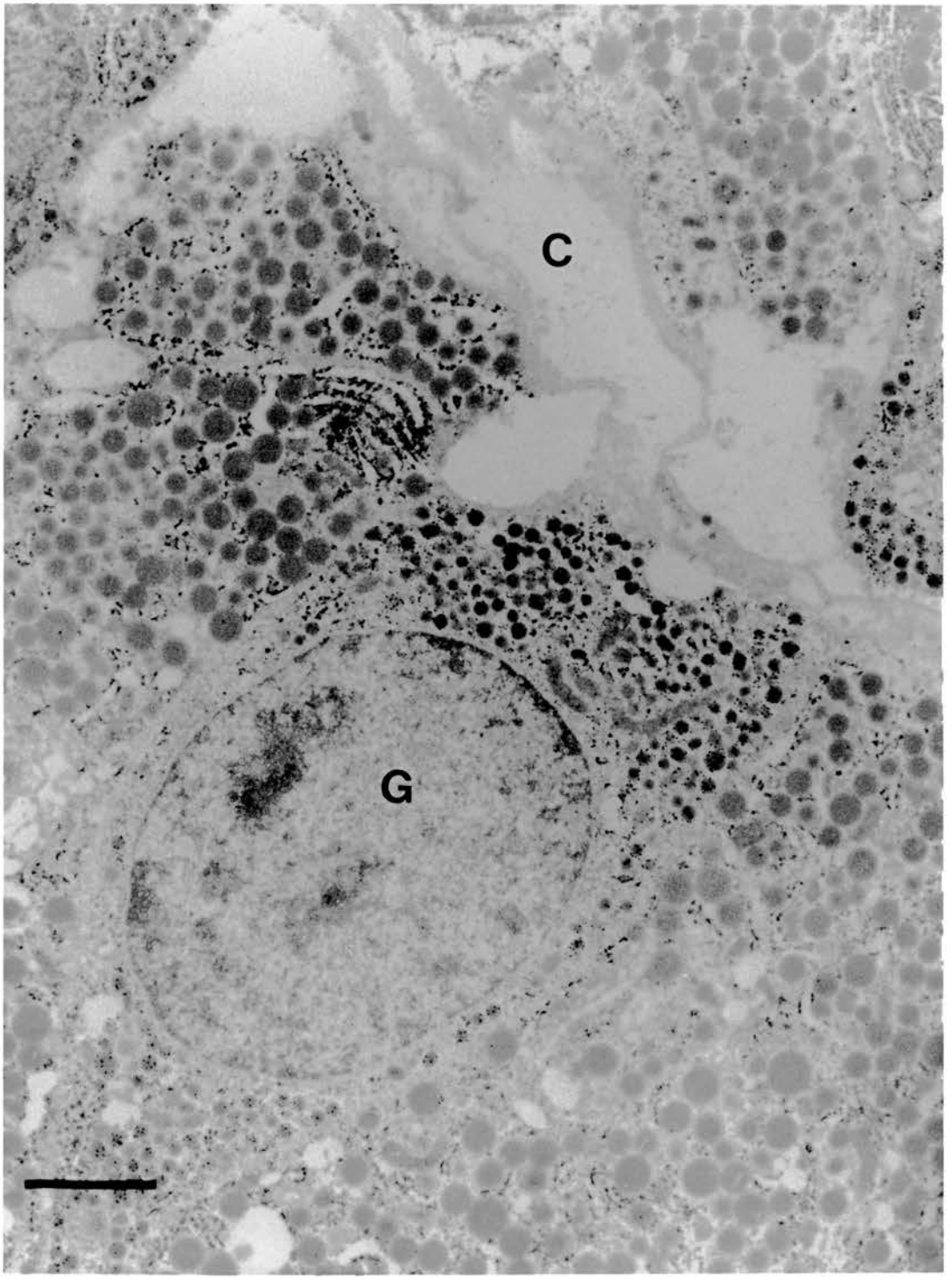
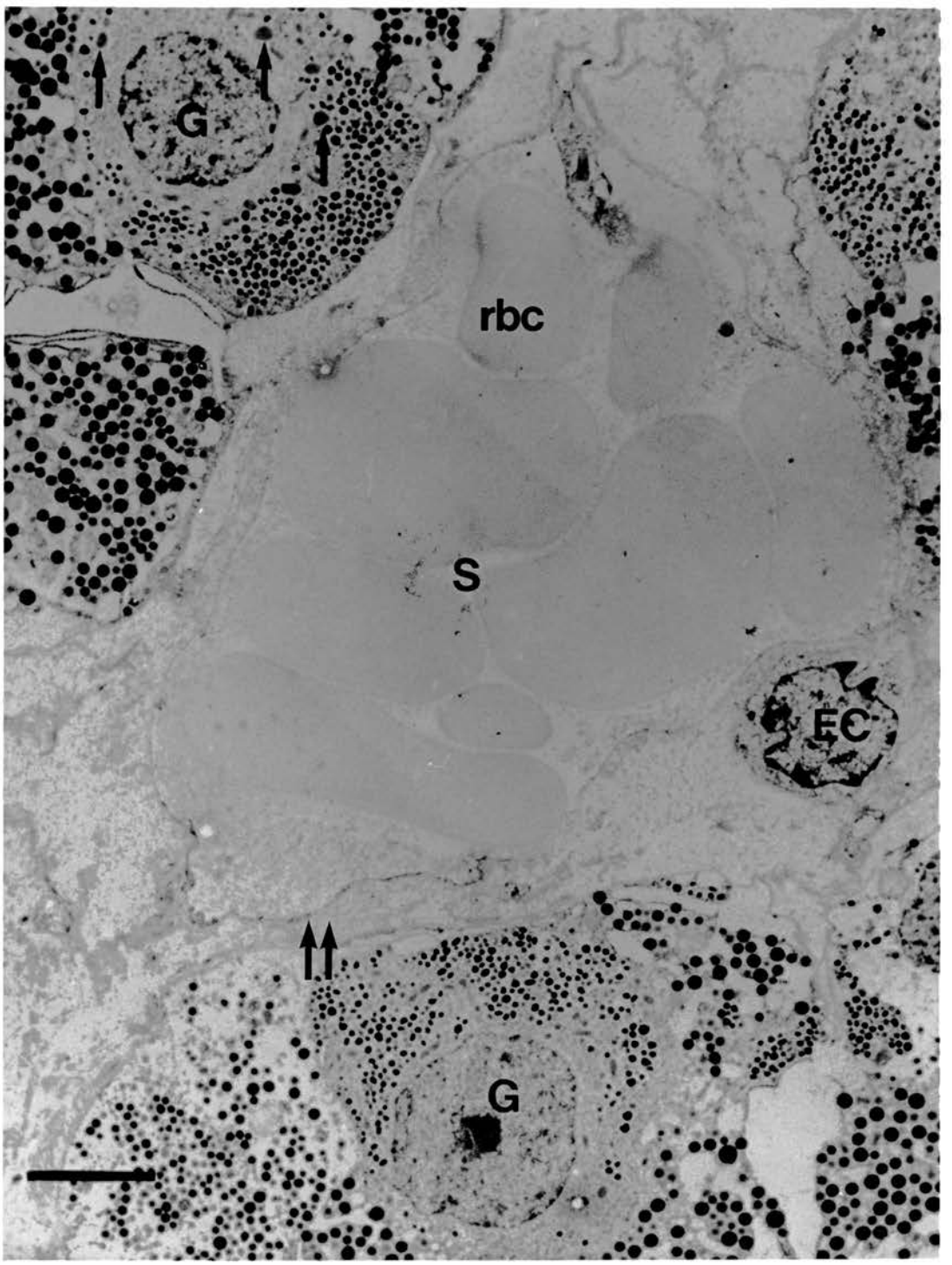

C
G

Figure 37. Transmission electron micrograph showing immunoidentified gonadotrophs (G) at oestrus plus 9h before the preovulatory LH surge. The gonadotrophs were located next to a sinusoid (S) lined with a fenestrated endothelium (↑ ↑) (see also Fig. 62) and containing red blood cells (rbc). An endothelial cell nucleus was present (EC). The majority of secretory granules were located in the aspect of the cytoplasm abutting the sinusoid (S) leading to the appearance of a polarised cell. Light dense bodies (↑) displayed a perinuclear distribution in one of the gonadotrophs. Magnification = 4900X. Scale bar = 4.1μm.


G
rbc
S
EC
G

gonadotroph population. Prior to the LH surge, granules appeared to be excluded from some cortical regions of the cell. During the buserelin-induced LH surge, 90% of gonadotrophs were polarised.

At the peak of the LH surge, a small number of secretory granules were observed in the cell periphery (Fig. 38). Changes in percentages of polarised cells during late luteal and throughout the follicular phase are shown in Figure 39.

At oestrus plus 24h the gonadotrophs were almost totally devoid of secretory granules (Fig. 40). The rough endoplasmic reticulum (RER) was well defined with the few remaining LHβ positive granules confirming the cell as a gonadotroph. This was the only time during the cycle at which this morphological appearance was observed. In these cells, high LHβ immunoreactivity was observed in the vicinity of the RER. At higher power (Fig. 41) LHβ subunit was present in the RER lumen.

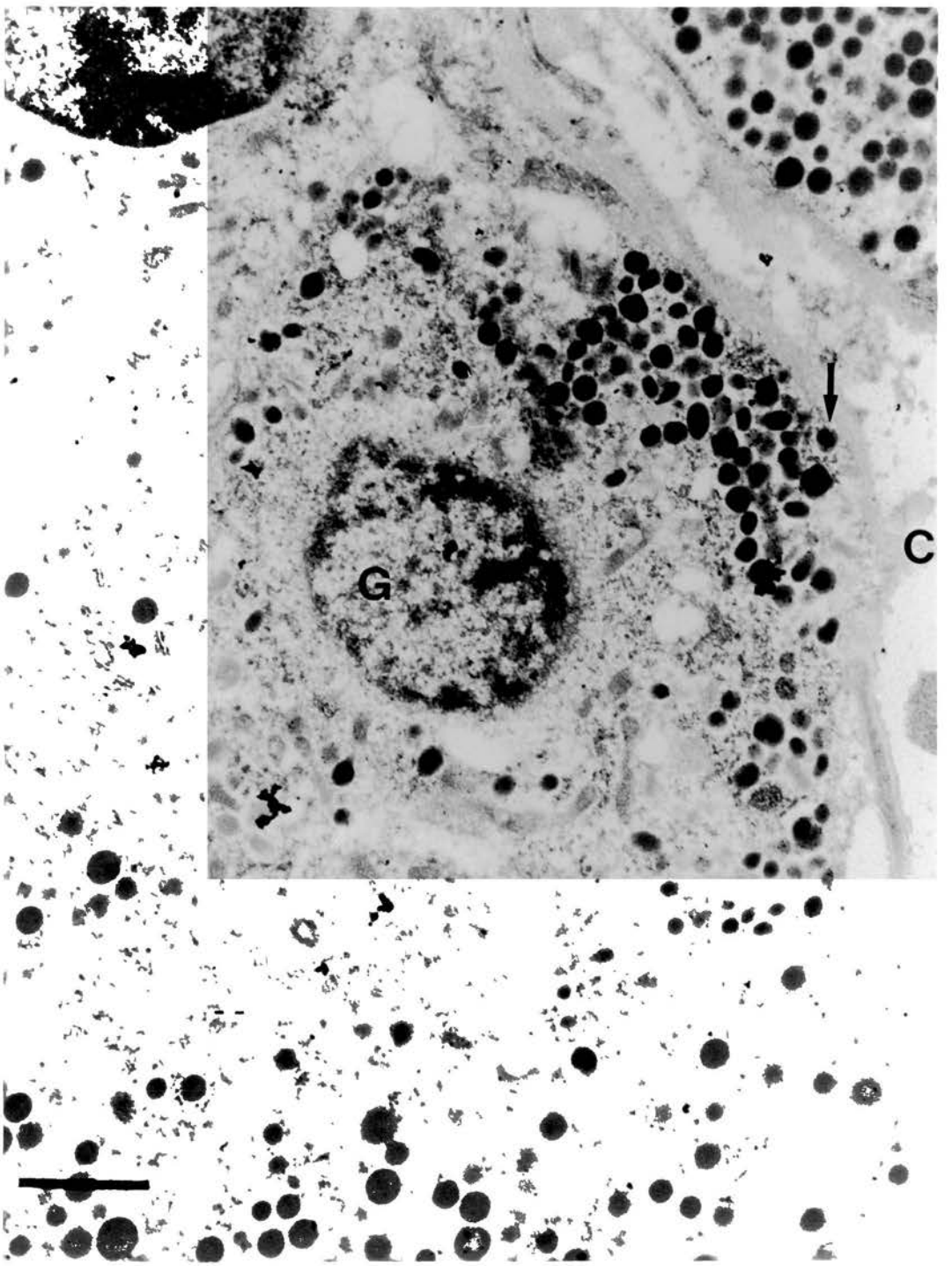
The animal in the oestrus plus 24h group which failed to exhibit a preovulatory LH surge, possessed many gonadotrophs which displayed a polarised morphology. However, the cortical regions of the cytoskeleton were noticeably thickened, the granules residing some considerable distance from the plasmalemma (Fig. 42). The percentage of polarised cells in this animal was similar to those observed in the buserelin treated group during the LH surge.

Light dense bodies (the term is taken from earlier observations on similar structures reported in the rat - Garner and Blake 1981) were found in very small numbers at all stages of the cycle. Their absence in the majority of cell profiles precluded any meaningful stereological evaluation. They appeared randomly localised throughout the cytoplasm, often failing to migrate with the main granule body (Fig. 36). Furthermore, they were found in cell profiles which were otherwise agranular. Thus, they have not been classified as secretory granules.

4.2.5. Stereology

In the four animals examined, maximal diameters of granule profiles lay between 300 and 309nm. Frequency distributions of profile diameter from gonadotrophs with non polarised and differing degrees of polarised granule distributions (Fig. 43) showed a large decrease in the 0.5 profile diameter class interval between the non-polarised morphology and the polarised morphology observed prior to the LH surge. This was

Figure 38. Transmission electron micrograph showing a gonadotroph (G) from the peak of the LH surge. LHβ immunopositive secretory granules were fewer in number and located in close proximity to the plasma membrane (↟). The granule distribution was still polarised towards the adjacent capillary (C). Magnification = 15000X. Scale bar = 1.3mm.


G
C

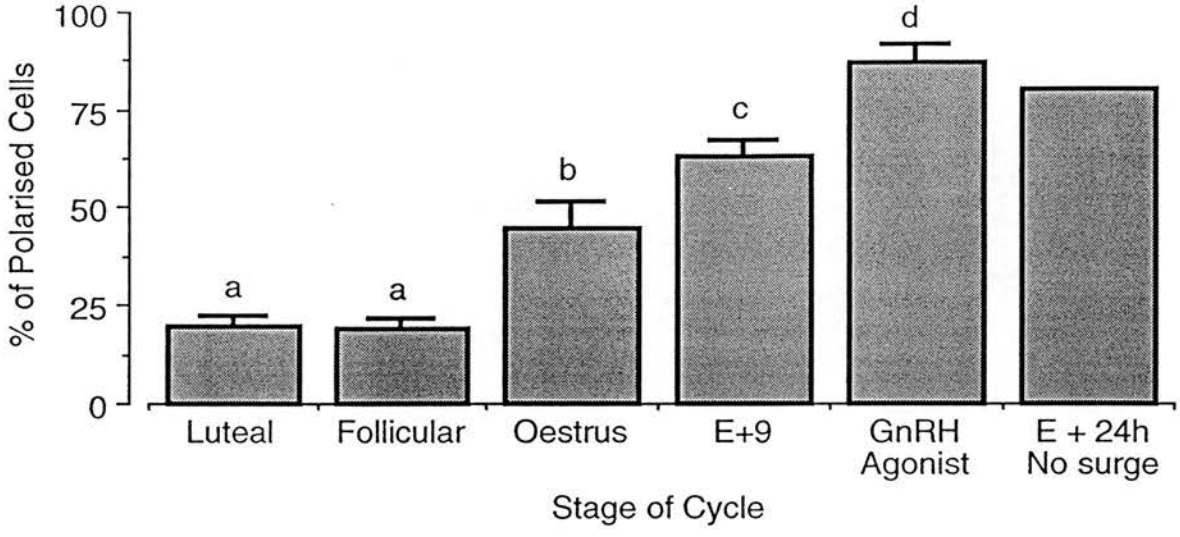


Figure 39. Changes in the percentage of polarised gonadotrophs from luteal through follicular phase to the LH surge. Values are mean ± SEM, n=5. Data was analysed by one-way ANOVA. Different letters denote significant ($P < 0.05$) differences.

Figure 40. Transmission electron micrograph showing a gonadotroph (G) at oestrus plus 24h. The presence of isolated LHβ immunopositive secretory granules (↟) confirmed the cell as a gonadotroph. At this time in the oestrous cycle, after the LH surge, the cytoplasm was predominantly agranular with the rough endoplasmic reticulum (RER) well defined. Magnification = 12500X. Scale bar = 1.6mm.

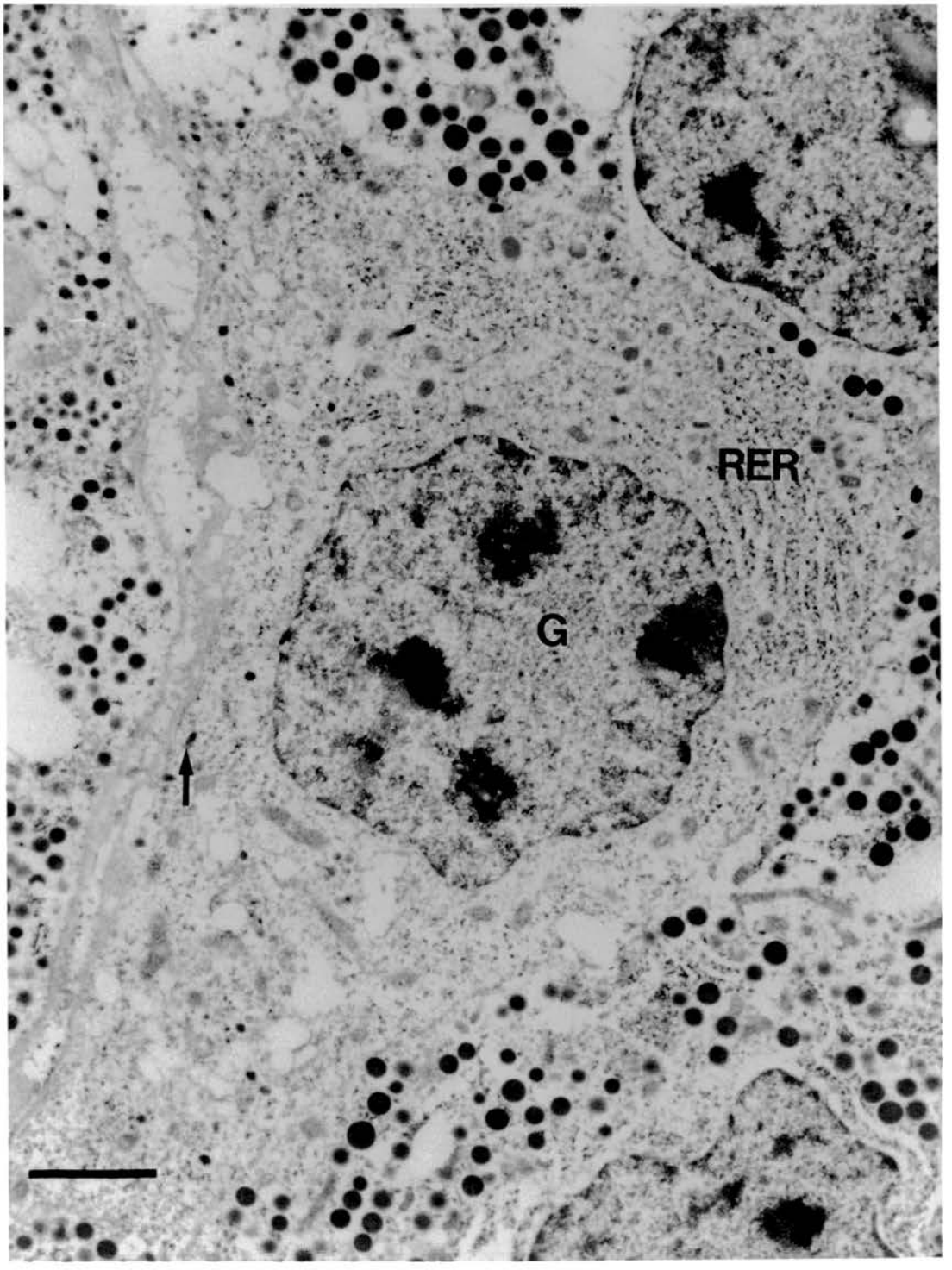

RER
G

Figure 41. Transmission electron micrograph showing the rough endoplasmic reticulum of a gonadotroph from oestrus plus 24h. LHβ subunit, immunolocalised using 15nm gold particles, was present in the lumen of the rough endoplasmic reticulum (RER) and in isolated secretory granules (↟). The ovoid LHβ immunopositive structures of low electron density (↟ ↟) may be bulbous protrusions of RER cut in cross-section. Magnification = 101000X. Scale bar = 200nm.

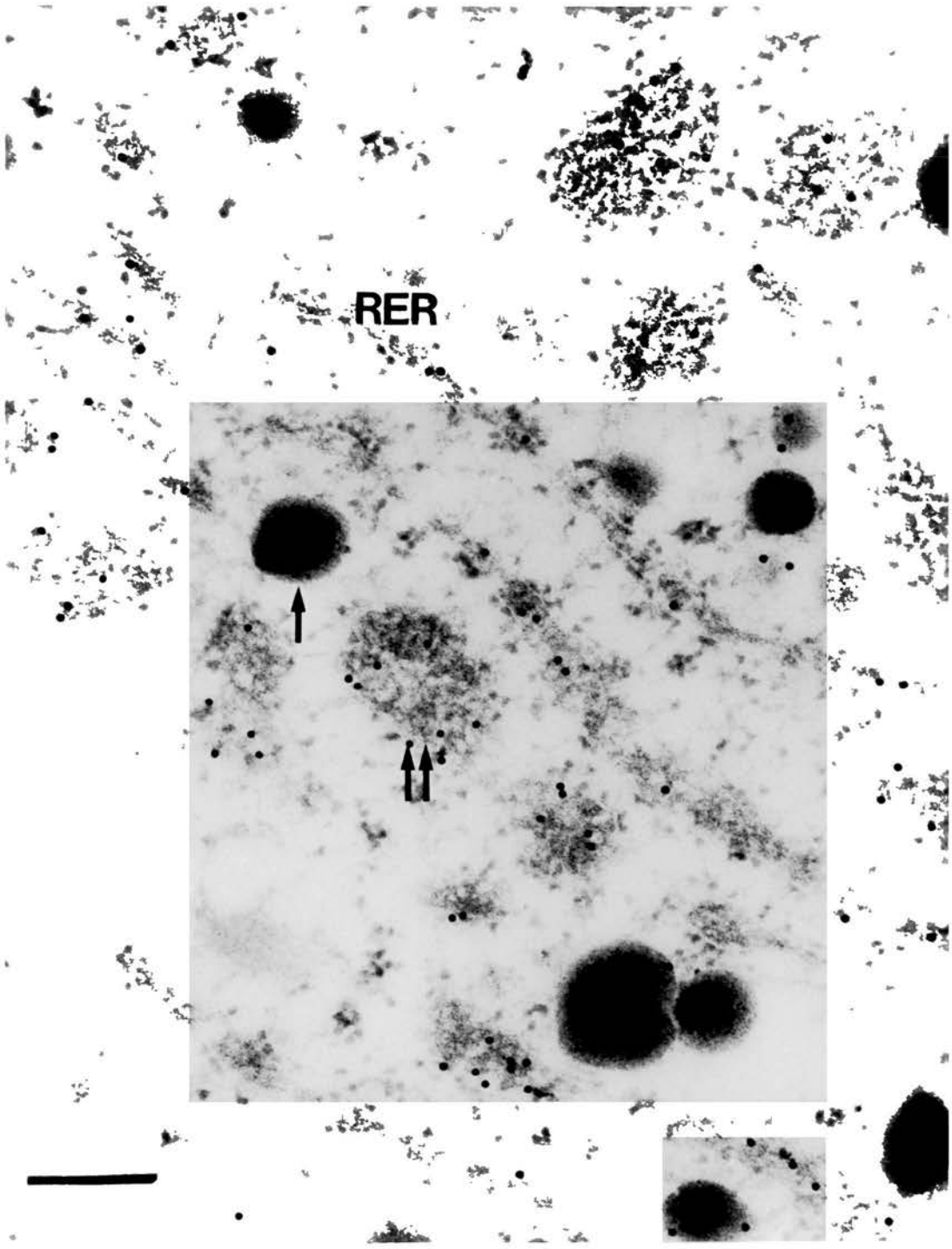
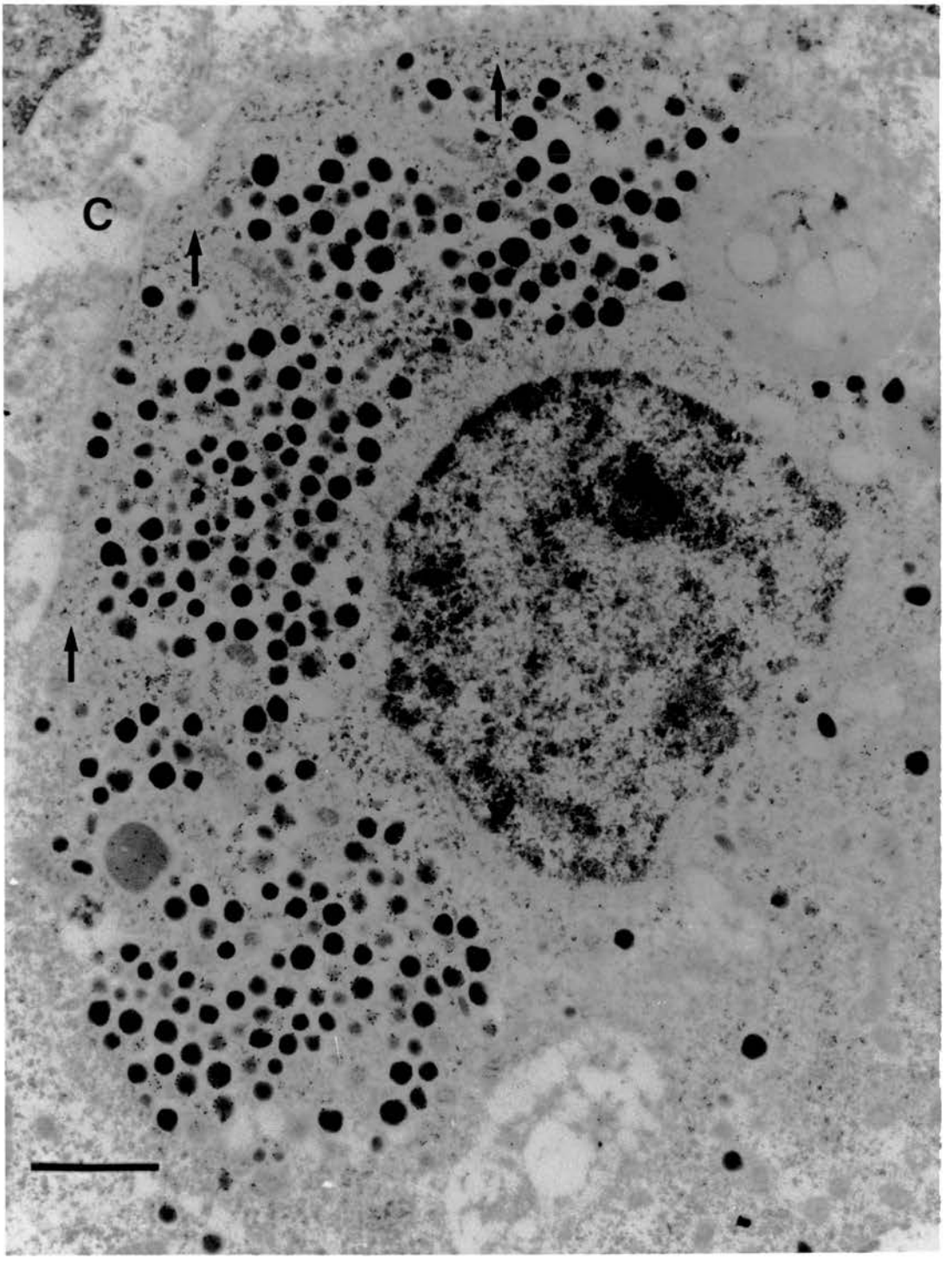

RER

Figure 42. Transmission electron micrograph showing an immunoidentified gonadotroph from the animal from the oestrus plus 24h group that failed to produce an LH surge. Although the distribution of secretory granules was polarised in the direction of the adjoining capillary (C), most granules were not in close apposition with the cell membrane. This gave the appearance of a thickened cell cortex (↟). Magnification = 21000X. Scale bar = 945nm.


C

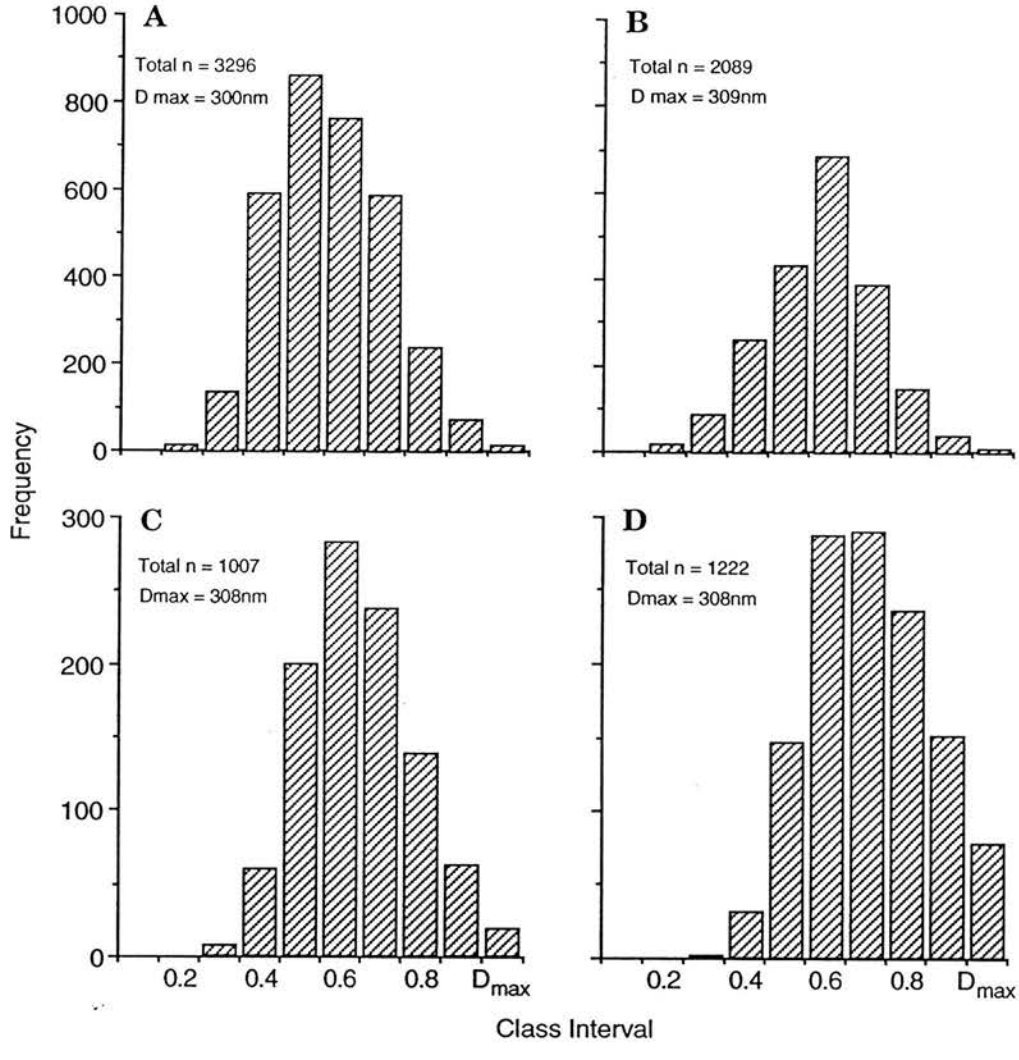


Figure 13. Comparison of the frequency distribution histograms of granule profile diameter class intervals from non polarised and polarised gonadotrophs. Non-polarised cells were randomly sampled from pituitaries in the luteal phase (A) and polarised cells were analysed from pituitaries immediately prior to the LH surge (B), at mid LH surge (C) and peak LH surge (D). D_{max} = maximum profile diameter (309nm). Class intervals are 10% increments of the maximum observed profile diameter.

reflected in a shift of the modal class from 0.5 in the non-polarised cells to 0.6 in the pre-LH surge polarised cells. In gonadotrophs exhibiting extreme degrees of polarised granule distributions, during the LH surge, the modal profile diameter class interval shifted from the 0.6 class at mid-LH surge, to the 0.7 class at peak LH surge.

The shifts in modal class in the frequency histograms were confirmed when numbers of observations in each class interval were expressed as percentages of the total number of observed profiles (Fig 44).

When the observed number of profiles were converted to actual numbers of granules/mm^3 of cytoplasm using the Schwartz-Saltykov diameter analysis, classes 0.1, 0.2 and 0.3 were devoid of granules in all cases (Fig. 45). The 0.4 granule diameter class was absent from the polarised gonadotrophs in mid-LH surge, whilst the 0.4 and 0.5 diameter size classes were absent in cells with extreme granule polarisations at peak LH surge. Shifts in the modal size class paralleled those described in the frequency and percentage histograms (Figs. 43 and 44). Total numbers of granules/mm^3 of cytoplasm and cytoplasmic areas per cell profile are given in Table 3. Mean gonadotroph cytoplasmic area showed a significant ($P<0.05$) increase in polarised cells before the onset of the LH surge.

Table 3. Summary of data from the Schwartz Saltykov diameter analysis showing the total cytoplasmic concentration of granules from all size class and gonadotroph cytoplasmic area (mean ± SEM). 1, n = 20 cells; 2, n = 13 cells. Differences in cytoplasmic area were analysed by one-way ANOVA and * denotes a significant ($P<0.05$) difference.

Cell Morphology	**Total Number of Granules ($x10^9/mm^3$ cytoplasm)**	**Gonadotroph Cytoplasmic Area (mm^2)**
[1]Non-Polarised - Luteal Phase	20.57	48.16 ± 3.12
[1]Polarised - Pre LH Surge	8.57	66.88 ± 5.55*
[2]Polarised - Mid LH Surge	16.18	34.53 ± 17.00
[2]Polarised - Peak LH Surge	10.77	47.18 ± 3.32

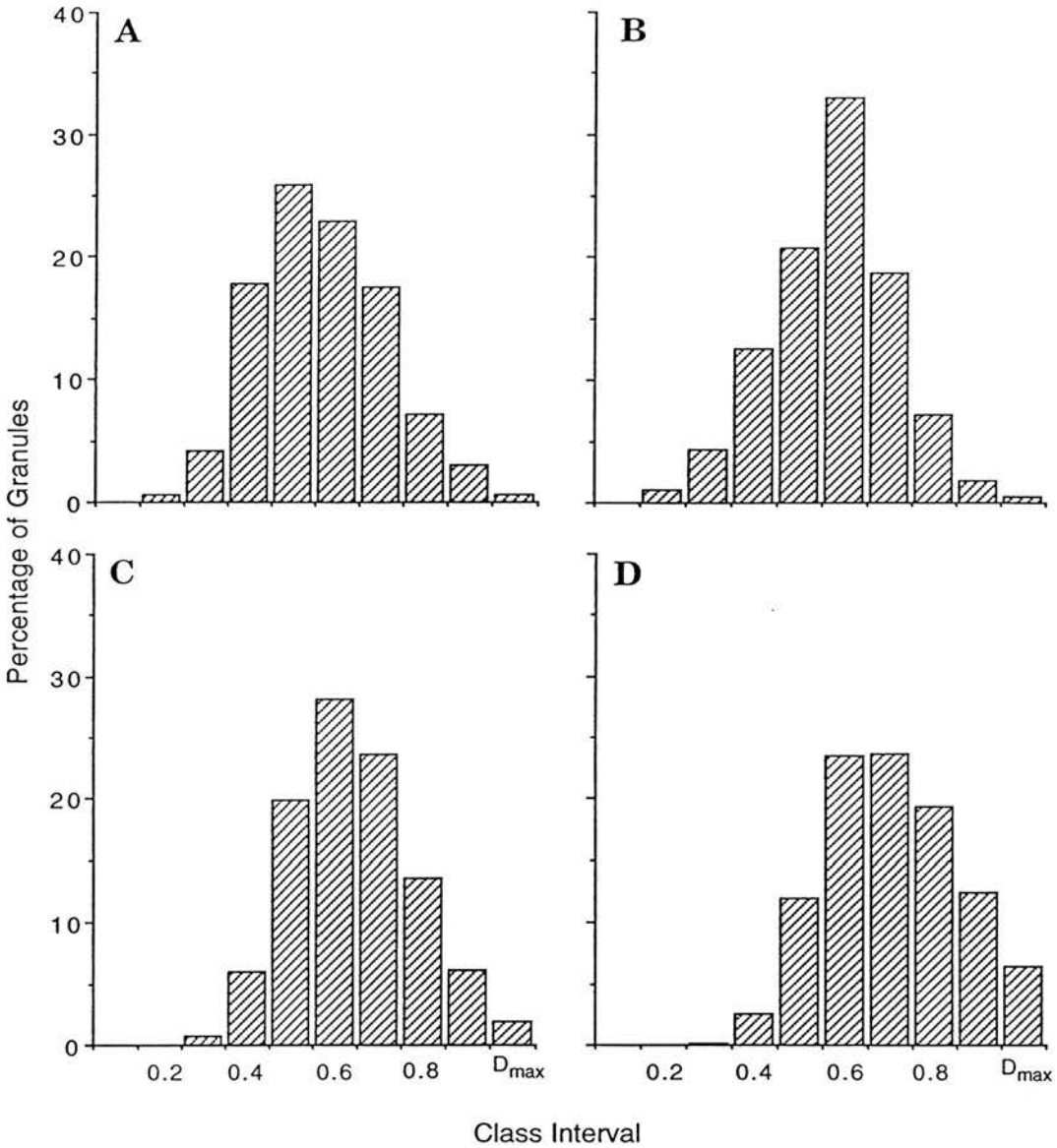


Figure 44. Comparison of the percentage distribution histograms of granule profile diameter class intervals from non-polarised and polarised gonadotrophs. Non-polarised cells were randomly sampled from pituitaries in the luteal phase (A) and polarised cells were analysed from pituitaries immediately prior to the LH surge (B), at mid LH surge (C) and peak LH surge (D). D_{max} = maximum profile diameter (309nm). Class intervals are 10% decrements of the maximum observed profile diameter.

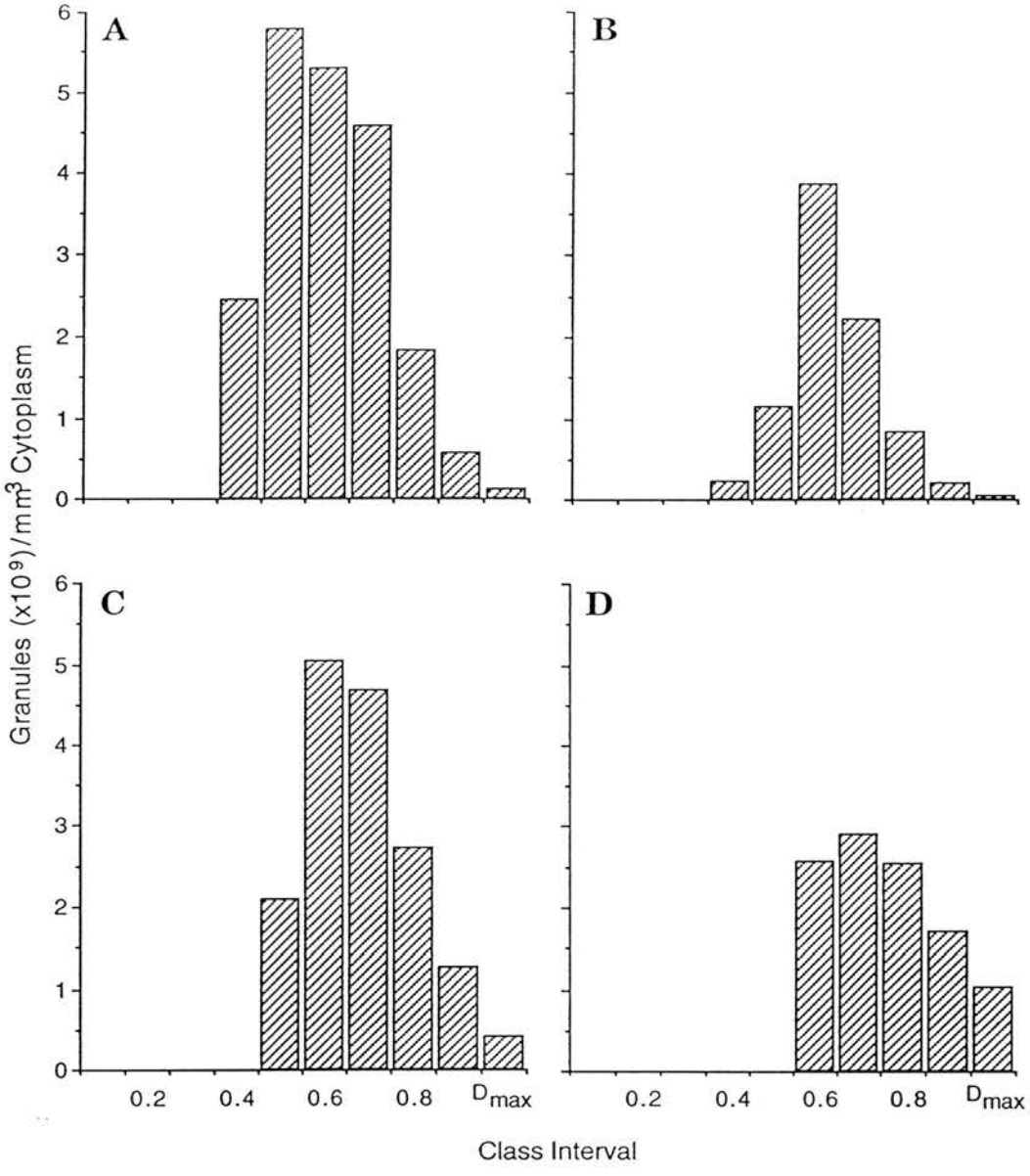


Figure 45. Comparison of Schwartz-Saltykov diameter analysis distributions showing numbers of granules $(10^9)/mm^3$ of cytoplasm in non-polarised and polarised gonadotrophs. Non-polarised cells were randomly sampled from pituitaries in the luteal phase (A) and polarised cells were analysed from pituitaries immediately prior to the LH surge (B), at mid LH surge (C) and peak LH surge (D). D_{max} = maximum granule diameter (309nm). Class intervals are 10% decrements of the maximum granule diameter.

4.3. Discussion

In the ovine gonadotroph, secretory granules were polarised to the region of the cell subjacent to the vascular system prior to exocytosis. The percentage of polarised cells increased during the follicular phase, reaching a peak value in mid LH surge. A simultaneous decrease in LHβ mRNA occurred between luteal phase and oestrus suggesting that the preovulatory LH surge is not related to increased synthesis of LH but a progressive recruitment of gonadotrophs into a releasing state as indicated by the polarisation of secretory granules towards the abutting capillary. The decrease in LH pituitary content which occurred from early follicular phase to oestrus may be a reflection of the increased LH pulse frequency which is present in the late follicular phase (Baird 1978; Wallace *et al* 1988). At oestrus plus 24h, following the LH surge, the appearance of LHβ immunoreactivity in the RER lumen and granular structures of low electron density, in a predominantly agranular cytoplasm, indicated the initiation of the replenishment of intracellular LH stores.

The above findings contrast with the data on the rat oestrous cycle. Rat gonadotrophs exist as small, medium and large sized subsets, with an increased proportion of the large cell moiety evident as the animal passes from pro-oestrus to oestrus (Childs *et al* 1992a). The small population are monohormonal - containing LH or FSH - whereas the large subset are predominantly multihormonal - containing LH and FSH (Lloyd and Childs 1988). During the approach to the preovulatory LH surge, LHβ transcription rates increase from the morning of di-oestrus through the afternoon of pro-oestrus, reaching a peak at 1700h (Shupnik *et al* 1989a). Zmeili *et al* (1986) have demonstrated two peak levels of LHβ: one between 0800-2000 in di-oestrus and the other on the afternoon of pro-oestrus just prior to the onset of the LH surge. This pre-surge upregulation in total expression is attributable to increased mRNA levels in both existing large cells, and small gonadotrophs which have enlarged or increased their density (Childs *et al* 1992a).

Analogies between the sheep and the rat are possible when the 'LH releasable pool' theory is considered. In the rat, the number of gonadotrophs able to release LH increases by a factor of two from di-oestrus to pro-oestrus, as determined by reverse haemolytic plaque assay (Smith *et al* 1984). The non-secretory cells lack GnRH receptors (Smith and Neill 1987; Neill *et al* 1987). The same authors believe the 'non-readily releasable pool of LH' to be located in the non-secretory gonadotrophs. An alteration of cellular function, transforming these cells to the secretory moiety, would

allow the transfer of LH into the 'releasable pool'. This transfer may be mediated by a cholera toxin-sensitive guanyl nucleotide binding protein. An 18h incubation of dispersed pituitary cells with cholera toxin leads to increased LH release when the cells were challenged with continuous or pulsatile nanomolar GnRH (Janovick and Conn 1993). The binding protein may act at the filamentous actin cortex and within the microtubule system: cholera toxin administration to cytotoxic lymphocytes disrupts filamentous actin patch formation and interferes with the orientation of the microtubule organising centre (Sugawara *et al* 1993).

The present observations in the sheep suggest that the non-polarised gonadotroph contains the 'non-releasable pool of LH', whilst polarisation allows LH to become releasable. It has been demonstrated that GnRH-binding activity and GnRH mRNA increase significantly from luteal levels at around 30h before the preovulatory LH surge (Brooks *et al* 1993). The acute nature of this rise over a period of 24h and the fact that levels are not significantly different 8h before the surge means that the increase does not correlate exactly with the gradually increasing percentages of polarised cells present as the animal nears the LH surge (Fig. 39). It is still possible that polarised gonadotrophs may be the subset that express the GnRH receptor and the degree to which it is expressed varies with the extent of polarisation. The overall picture may be further complicated as the progressively emptying waves of gonadotrophs may down-regulate their GnRH receptor expression whilst polarising cells may upregulate the same receptor. Differences in overall numbers and rates of change will contribute to the overall pituitary expression as determined by Northern analysis of the total RNA. Current data is therefore inconclusive when attempting to describe the changes occurring with an individual cell throughout the ovine oestrous cycle.

Further studies using *in situ* hybridisation at the level of the electron microscope are required to determine if the polarised form of the gonadotroph expresses GnRH receptor mRNA and hence the GnRH receptor.

In the rat, the cell type primarily responsible for the generation of the LH surge has not been determined exactly. Using perifusion apparatus, it has been shown that the extent to which entire dispersed pituitary glands respond to GnRH increases biphasically during pro-oestrus at both 0800h and 1400h (Evans *et al* 1983). Although the large gonadotroph enriched cultures show increased responsiveness to GnRH on the morning of pro-oestrus, this increment in lost by afternoon pro-oestrus (Childs *et al* 1992b). The same authors suggested that this decreased function may be attributable

to the absence of paracrine influences from the small and medium sized gonadotroph subpopulations in these enriched culture preparations. An increased LH responsiveness to GnRH in the small and medium sized cell fractions, observed at pro oestrus, may contribute to the preovulatory LH surge.

Although no part of this experiment was specifically designed to investigate changes in cell size, parallels do exist between the observations in the rat and the sheep. Polarised gonadotrophs have been found, in varying amounts, at all stages of the cycle. The increase in their number as the animal approaches and enters the preovulatory LH surge is indicative that a polarised morphology may be associated with exocytosis. The non-polarised cell, being most prevalent during luteal and early follicular phase when mRNA levels are at a peak, is likely to be the active synthesizing and storage form of the gonadotroph. This is similar to the function tentatively assigned to the small cell subpopulation in the rat.

The cytoplasmic areas used in the Schwartz Saltykov diameter analysis suggest that cell size may increase from luteal to follicular phase. Further studies on volume changes of gonadotrophs throughout the oestrous cycle are required to confirm this observation.

The low percentage of polarised cells in luteal and early follicular phase would allow the animal to generate the low amplitude LH pulses typical of this stage of the cycle. The gradual recruitment of the non polarised moiety into the polarised state coincides with an increase in LH pulse frequency. The time of maximum exocytotic activity, the LH surge, correlates with the highest percentage of polarised cells. Thus it appears that exocytosis from polarised cells satisfies the increased total requirement for LH at this time in the cycle. The recruitment of polarised cells in the sheep may be analogous with the enlargement of small gonadotrophs to the larger form as the LH surge approaches in the rat.

Previous ultrastructural observations in the rat and mouse have demonstrated the existence of two types of gonadotroph, known as type I and type II (Tougard and Tixier-Vidal 1988). Type I contain predominantly larger secretory granules (ca 300nm) of variable electron density which mature to the smaller dense core granules (ca 150nm) typical of the type II gonadotroph. GnRH induced exocytosis occurs only from the type II cells (Durnin and Morris 1992b). In this study, no evidence was found that morphologically different subtypes of gonadotrophs exist in the midregion of the ovine

adenohypophysis. The fact that no large granules of variable electron density were observed, suggests that the granule maturation process present in the rat does not occur in the sheep. Furthermore, the absence of granule fusion figures in the vast number of gonadotrophs viewed indicates that the larger granule classes do not appear to cleave to form smaller ones. Conversely, no evidence exists that small granules merge to form larger ones. It therefore appears that, in the sheep, the size of a granule is determined once it buds off the Golgi dictyosome.

Exocytotic figures from GnRH-stimulated rat gonadotrophs *in vitro* have been measured at 150nm using a tannic acid fixation technique (Durnin and Morris 1992a). The stereological investigations in the present study in sheep have demonstrated initial exocytoses centred around granules of 130-150nm in diameter as the non-polarised luteal phase gonadotroph becomes polarised at oestrus plus 9h. The further loss of smaller granules lead to the modal size class increasing from 130-150nm in luteal phase, to 180-210nm by the peak of the LH surge. Since no data is available with regard to events after the peak of the preovulatory LH surge, the exact fate of the largest size classes of granule remains unknown. Clearly, they are exocytosed in some form as demonstrated by continued LH plasma secretion and the presence of empty cells at oestrus plus 24h. Two broad possibilities exist: (i) the larger granules condense, forming smaller ones which are then exocytosed this requires a procedure that has not been observed at any stage of the ovine oestrous cycle in this experiment; (ii) the size-dependent exocytosis continues with the 300nm granules eventually exiting the gonadotroph. The observations do indicate that the largest granules are only released at the time of the LH surge, this being the only time at which agranular cell profiles are evident.

The physiological significance of the apparent release of the largest granules solely during the preovulatory LH surge is unclear. It may provide a mechanism by which the nature of the LH secreted at this time may differ from that which composes LH pulses.

The use of chromatofocusing to investigate the charge heterogeneity of ovine LH has demonstrated the existence of eight isoforms of the hormone in pituitary extracts which have been designated A-G and Z respectively (Keel *et al* 1987). In one instance, 13 isoforms were isolated (Zalesky *et al* 1992). Immunization against GnRH has been shown to increase the percentage of LH present as isoform F in rams and wethers (Zalesky *et al* 1993), whilst castration shifts the entire distribution towards the acidic isoforms (Keel *et al* 1987). Sexual maturation has been shown to have no effect on

isoform distribution in bovine pituitary glands, but ovariectomy leads to a decrease in the more basic isoforms which is reversed with oestradiol administration (Stumpf *et al* 1992). Ovine intrapituitary LH isoforms have been shown to remain relatively constant between late follicular phase, mid-luteal phase and anoestrus (Zalesky *et al* 1992). However, the same authors did report increased proportions of the weakly basic and acidic moieties during anoestrus. Isoform F, despite the decreased GnRH input during anoestrus, did not differ significantly between treatments. In the horse, the LH isoforms secreted are more alkaline at oestrus than those typically stored in the gland (Shand *et al* 1991). The same authors suggested modification of the product after its transfer into a preferentially released pool.

The physiological significance of the existence of differing LH isoforms has not been determined. The ovarian LH receptor may require different LH isoforms of different half lives to prolong or shorten the effects on ovarian steroid synthesis. The investigations thus far undertaken have concentrated on the content of the entire pituitary. It is possible that all isoforms are synthesized simultaneously when the cell is in the non-polarised state. The preferential release of a different form of LH as reported in the horse could be achieved by the preferential exocytosis of a granule subset at a suitable time during the oestrous cycle. In order to test this hypothesis, greater study of the LH exocytosed into the portal supply is required. An accurate determination of the changes throughout the oestrous cycle together with ultrastructural immunocytochemical evidence of the localisation of the different isoforms between granule subpopulations may assist in the assignment of a physiological function. Alternatively, the acidic and basic isoforms may simply be a reflection of the isoelectric points required for the protein to package colocalised with e.g. the granins. Thus the isoforms may have no role once the granule exits the Golgi complex.

The numbers of granules/mm^3 of cytoplasm as determined by the Schwartz-Saltykov diameter analysis requires careful consideration. The values plotted in the histogram are in the form of concentrations. Although there is a drop in the total numbers of granule profiles as the animal passes through follicular phase to the LH surge, the concentration of granules appeared to decrease from luteal phase in the non polarised gonadotroph to oestrus plus 9h in the polarised gonadotroph. The increased degree of polarisation present in the mid LH surge appeared to accompany a further increase in granule concentration prior to a final decrease in cells exhibiting extreme polarisation (Fig. 45). These changes in granule concentration may be influenced by the increased

cytoplasmic profile area present immediately prior to the LH surge (Table 3). In cells exhibiting the most extreme degrees of polarisation, observed cytoplasmic areas returned to values similar to those observed in the non-polarised cells. It is possible that this area change was caused by a reduction in cell volume due to exocytosis of a major proportion of the total cytosolic content. The increase in cytoplasmic area may be required to initiate the LH surge and related to the increased responsiveness to GnRH shown by large gonadotrophs in the rat (Childs *et al* 1992b).

The other striking feature of the Schwartz-Saltykov plots is the apparent increased concentrations of the largest granule sizes at the time of the LH surge. Since the observed maximum granule diameter remains constant, these observations are not due to the synthesis of a larger granule population. The lack of any observed fusion figures suggests that small granules do not merge to form larger ones. The probable explanation lies in a consideration of the changes in localisation of granules throughout the cycle. By the time of the LH surge, all secretory granules are polarised to one aspect of the cell. The absence of any large gaps between granules and their progressively closer apposition with the plasma membrane indicates that the exocytosis of smaller granules allows the larger classes to move closer together. This change in relative localisation increases the probability of sectioning a large profile, thus resulting in the increased number of observations (see **2.2.6.4.**).

The explanation for the size-dependent nature of the exocytosis may involve the cortical regions of the cytoskeleton. Microfilaments and microtubules have been implicated in the control of secretion of insulin granules from β cells in the islets of Langerhans of the pancreas for many years (Lacy *et al* 1968). In dispersed ovine pituitary cells, disruption of the microfilament network reduces GnRH-stimulated LH release, whilst microtubule perturbation has no effect (Adams and Nett 1979). This lack of effect of the microtubule system may reflect the absence of polarisation of the granules, the dispersed cell being unable to orientate itself towards a vascular supply. Alternatively, granule transport may be achieved solely by the microfilaments. During GnRH priming using mouse pituitaries *in vitro*, the gonadotroph microfilaments changed orientation such that the incident angle between the filament and the plasma membrane was reduced (Lewis *et al* 1985). The introduction of cytochalasin B to disrupt microfilaments prevented the GnRH priming effect and significantly increased LH release in the first hour of GnRH exposure. This suggests that the microfilaments may act both as granule transporters and as a barrier preventing access to subplasmalemmal exocytotic sites whilst the priming effect takes place. Whilst physiological levels of extracellular

calcium are necessary for LH release, changes in extracellular calcium concentrations do not interfere with the priming response (Pickering and Fink 1979). It has been demonstrated in granule-containing chromaffin cells that the disassembly of the cortical filamentous actin network facilitates exocytosis. This disassembly of actin is mediated by the mobilisation of the protein scinderin, which is also abundant in the adenohypophysis (Trifaro 1992, Trifaro *et al* 1993).

It is possible that GnRH, via an elevated intracellular calcium concentration, is able to mobilise scinderin from its intracellular stores. The initial disassembly of the cell cortex would allow access of the smaller granule sizes to subplasmalemmal binding sites, thus leading to exocytosis. The continuation of the stimulus through the increased GnRH pulse frequency as the animal approaches the LH surge (Clarke *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994) would lead to greater filamentous actin disassembly, allowing the larger granule classes to interact with the membrane. In this way the largest granules would only be exocytosed at the times of the greatest stimulation (peak LH surge). Thus, a failure of the disassembly mechanism would prevent the LH surge. The expected morphological appearance would be one of thickened cell cortices as observed in the animal from the oestrus plus 24h group which failed to produce an LH surge. Microtubules may be involved at an earlier stage in the oestrous cycle during the transport of the granule body leading to polarisation. Their role in the generation of polarity in zoospores of Phytophthora cinnamomi has been demonstrated (Hyde and Hardham 1993).

These data suggest that, in the sheep, priming of the gonadotroph occurs via the polarisation of secretory granules to the region of the cell subjacent to the vascular system.

4.4. Granule Localisation Outwith the Nuclear Plane of Section

In all the morphological studies outlined in this chapter, polarisation of granules within the gonadotroph was assessed in the nuclear plane of section. To assess if granules remained polarised outwith the nuclear plane, a short serial section study was conducted.

4.4.1. Materials and Methods

A pituitary tissue block from oestrus plus 9h, used in the studies assessing polarisation, was sectioned serially and its ultrastructure assessed. A ribbon of seven ultrathin serial sections were cut and floated onto a gold slotted EM grid (Gilder), coated with Formvar (Agar Scientific) using a method described by Hayat (1970). Sections were immunostained for LHβ subunit to identify gonadotrophs. Following staining with saturated uranyl acetate and lead citrate, sections were examined in the transmission electron microscope. Gonadotrophs with polarised granule distributions in the nuclear plane were tracked across serial sections to determine if the localisation of the granule body remained constant despite the absence of a nuclear profile.

4.4.2. Results

Representative gonadotroph serial sections, from a pituitary immediately prior to the LH surge, (Fig. 46a and b) show that the polarised distribution of granules in the cytoplasm remains relatively constant despite the absence of a nuclear profile. Polarisation outwith the nuclear plane provided further evidence that movement of the granule body was a result of the translocation of majority of granules and not just those in the nuclear plane of section.

4.5. LHβ immunonegative granule profiles

The LHβ immunonegative granule profiles have not been reported in previous studies. As immunostaining is an effect upon the section surface, it was possible that these profiles represented either LHβ negative granules or planes of section through the granule from which LH was absent.

The way by which an electron microscope generates an image provides a different explanation. The electron beam descends the column and collides with the specimen (Fig. 17). The heavy metals used to stain the tissue (gold particle together with uranyl acetate [uranium] and lead citrate [lead]) deflect the electron beam which then does not impact on the photographic negative. This results in the production of a light area. Regions of the tissue which are free of heavy metals will not disrupt the passage of the beam and will therefore appear as dark areas on the negative. Upon printing, the light and dark regions are reversed to produce the familiar image. As the beam passes directly through the specimen, it is possible for a sphere to have an apparent diameter

Figure 46a. Transmission electron micrographs of serial sections through a polarised immunoidentified gonadotroph from oestrus plus 9h. In micrographs 1, 2, 3 and 4 the granule body (g) was located in the aspect of the cell nearest the adjacent capillary (C). The nuclear profile (N) gradually reduced in diameter as the serial sections progressed away from the nucleus. Magnification = 9100X. Scale bar = 2.1mm.


1
C


2


3

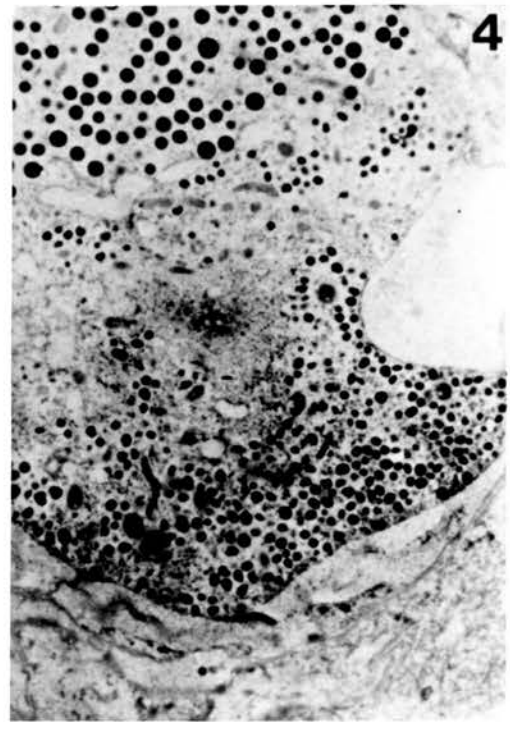
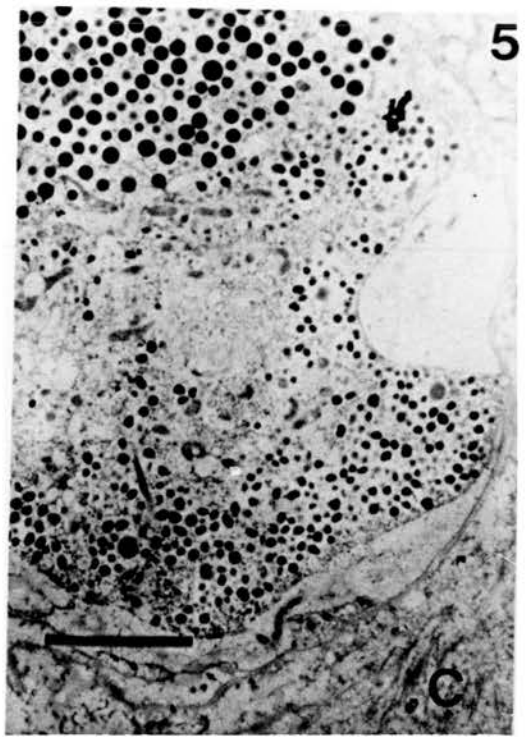
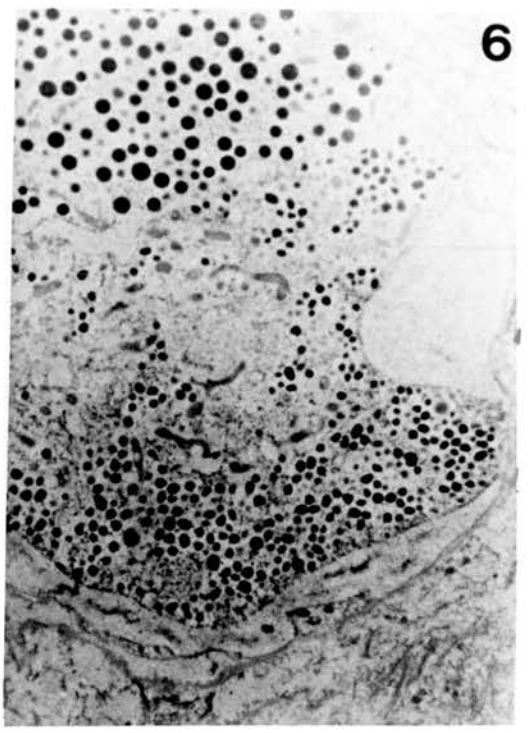

4

Figure 46b. In transmission electron micrographs 5, 6, and 7, the cell nucleus was outwith the plane of section. The granule body still exhibited the same polarised distribution towards the capillary (C). Magnification = 9100X. Scale bar = 2.1mm.


5
C


6


7

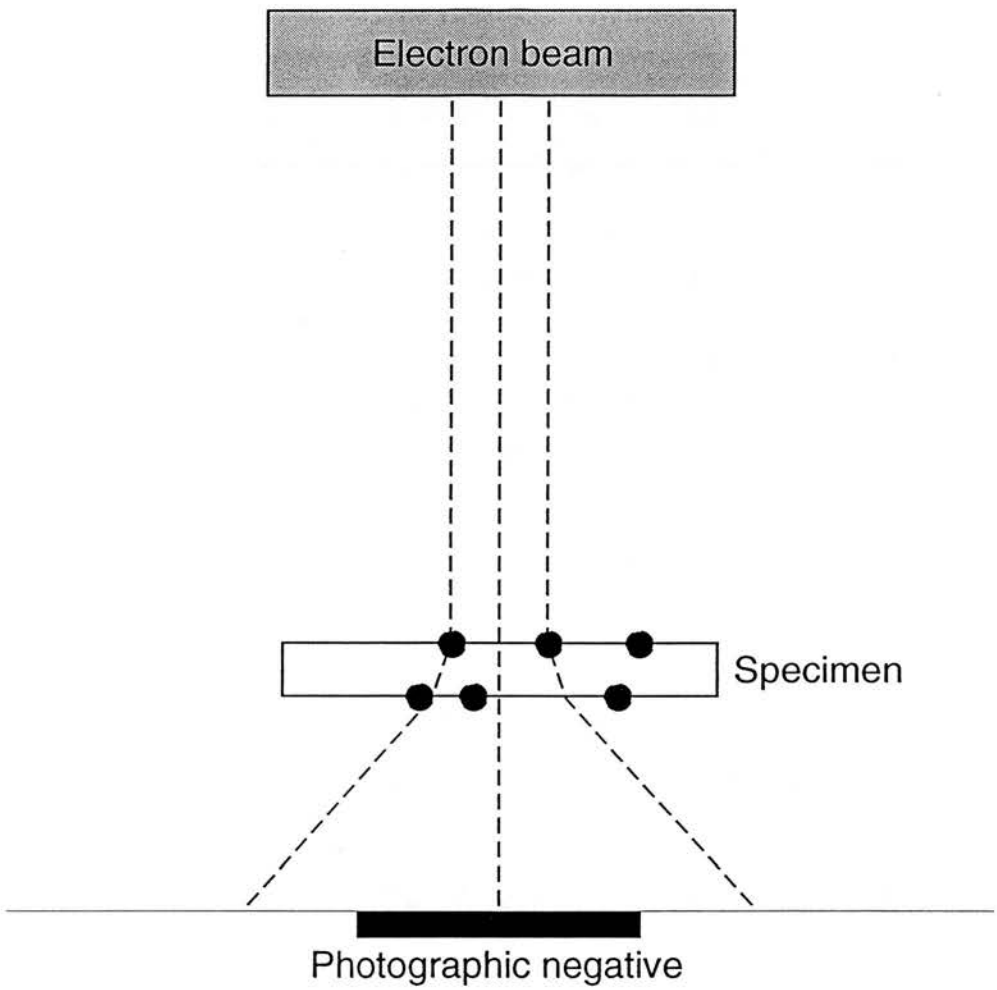


Figure 47. Diagrammatic representation of the generation of an image in the electron microscope. Deflection of the electron beam by tissue components and heavy metal stains prevent its collision with the negative and results in a dark area on the photomicrograph. Electrons which pass directly through the specimen create a light area on the micrograph.

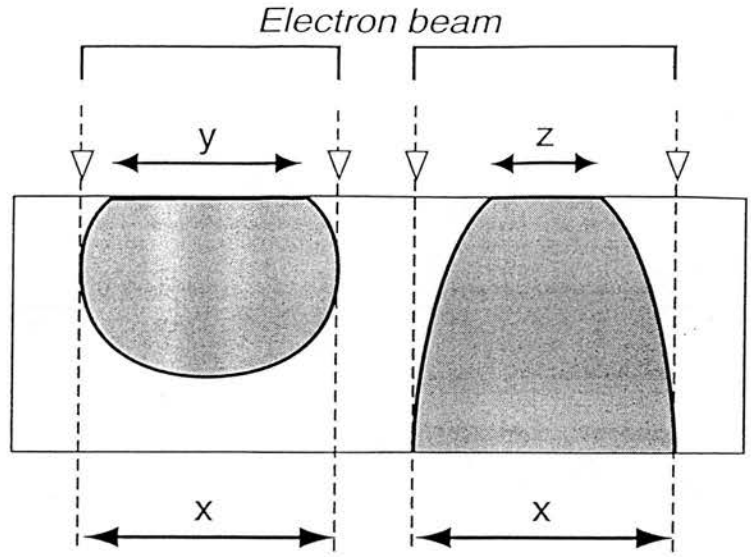
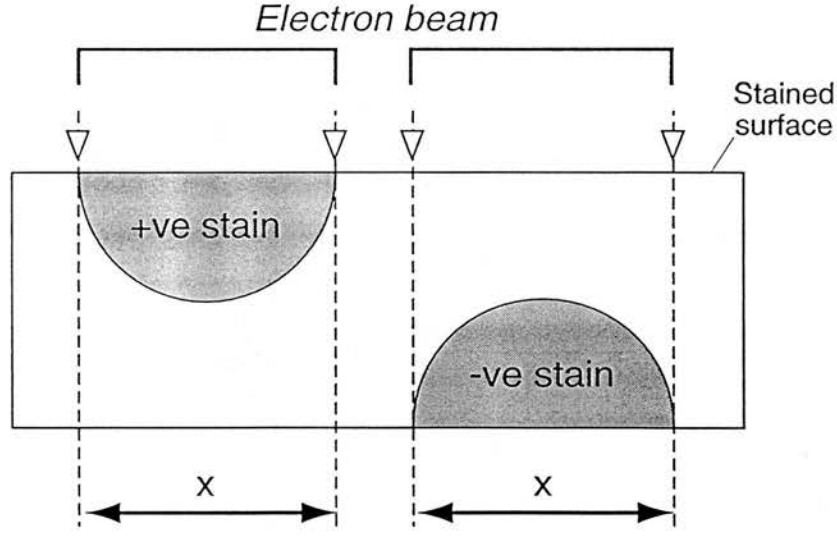


Figure 48 a) The Holmes Effect. Due to the deflection of electrons by the tissue components within the section, the area exposed at the section surface may be less than the actual observed area.



b) Diagrammatic representation of a mechanism for negatively stained granule profiles. A small granule profile may appear immunonegative because the cut surface is not exposed to the immunostain.

greater than that exposed at the sectioned surface. This is known as the Holmes effect (Fig. 48a). It is also possible for two spheres which have cut surfaces exposed at either side of the section, to be indistinguishable (Fig. 48b). Since immunostaining is a surface phenomenon, being carried out on one side of the section only, a positive profile may appear immunonegative.

4.5.1. Materials and Methods

Ultrathin sections of ovine adenohypophysis were stained for the presence of LHβ subunit as previously described. The sections were then reversed and the staining procedure repeated from the other side. Following staining with uranyl acetate and lead citrate, sections were viewed in the Phillipps TEM 400 electron microscope.

4.5.2. Results and Conclusion

All observed granule profiles were LHβ immunopositive (Fig. 49). At higher power, granule profiles showed an extremely high gold particle staining density as a consequence of the immunodetection of the antigen from both sides of the tissue (Fig. 50). Thus all granules in the ovine gonadotroph contain LH. It is theoretically possible for a small granule, with a diameter less than the section thickness, to expose no cut surfaces and hence appear immunonegative. The absence of immunonegative granule profiles following the staining of LHβ subunit from both sides of the section strongly indicates that the gonadotrophs in this study had no LH-containing granules smaller than approximately 60-90nm. This is confirmed by the stereological calculations in section **4.2.5.**

4.6. Pilot Studies on the Control of Polarisation

In every cell observed in the natural ovine oestrous cycle, the polarisation of the LHβ immunopositive granule body occurred in the direction of the vascular system. GnRH exerts a priming action on the pituitary gland and *in vitro* GnRH receptor mRNAs are increased by oestradiol and inhibin (Wu *et al* 1994). Since increasing oestradiol production *in vivo* from oestrogenic follicles correlates with the pre-LH surge rise in GnRH binding and GnRH receptor mRNA abundance (Brooks *et al* 1993), and GnRH receptor down-regulation post-surge relates to a sudden reduction in follicle oestradiol content and thus oestradiol secretion rate (Baird and McNeilly 1981, Baird *et al* 1991), oestradiol may control the direction and extent of polarisation. In an attempt to

Figure 49. Transmission electron micrograph showing the immunogold localisation of LHβ subunit stained from both sides of the section. All granule profiles were LHβ immunopositive. The almost complete absence of gold particles over the nucleus (N) indicated that background staining was extremely low. Magnification = 36000X. Scale bar = 555nm.

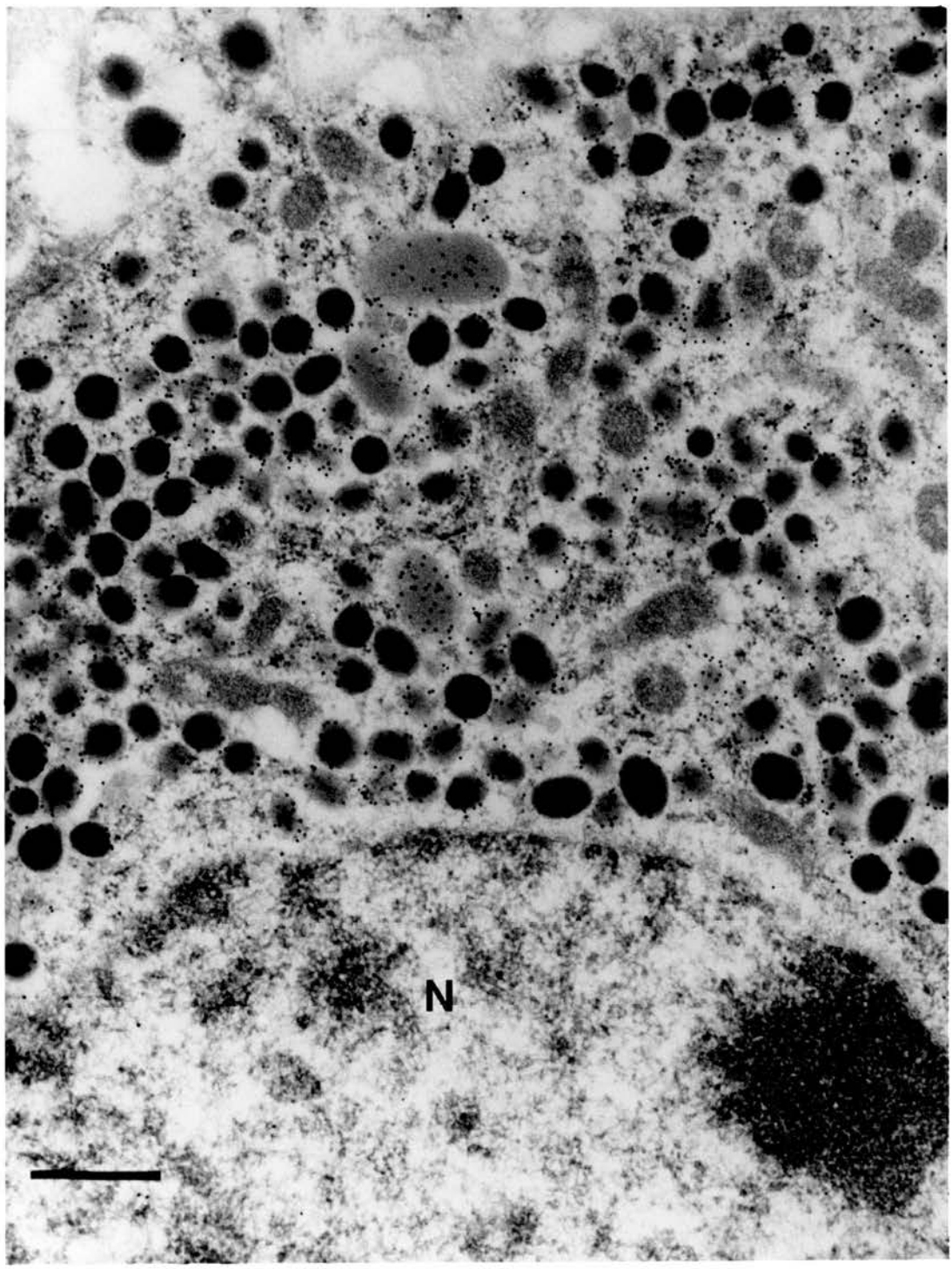
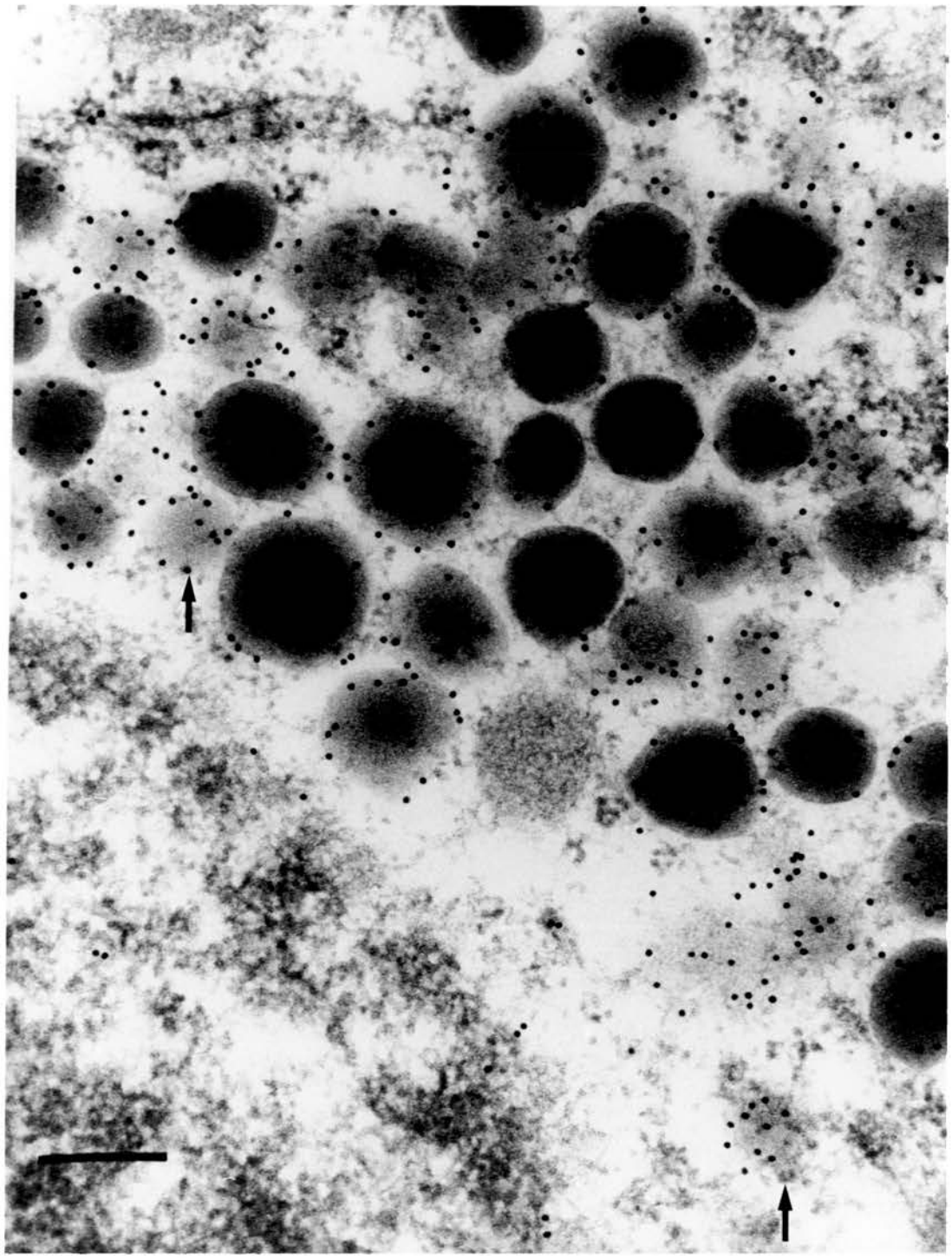

N

Figure 50. At higher magnification, LHβ staining, achieved by staining the section from both sides, was clearly more intense than the single side staining technique used previously (see Fig. 35). Small profiles and those with low electron densities (↟) were highly immunoreactive for LHβ subunit. Magnification = 98000X. Scale bar = 200nm.



determine a role for oestradiol in this process, a small study was conducted with six luteal phase ewes (where the non-polarised cell moiety predominates).

4.6.1. Materials and Methods

Following synchronisation of the oestrous cycle as previously described, three animals were treated parenterally with 100μg of oestradiol benzoate (a dose which in previous studies had been shown to produce an LH surge). Three untreated animals served as controls. Animals were killed 18h after oestradiol benzoate administration, the pituitaries removed and processed for immunoelectron microscopy. Sections were stained for the presence of LHβ subunit and visualized with 15nm gold particles. Differences in polarised morphology between the two groups were determined by counting 20 randomised cells per animal. Blood samples taken before and after oestradiol treatment were assayed for LH to determine the endocrine status of the animal.

4.6.2. Results

Oestradiol administration induced the polarisation of 75±0% of gonadotrophs in the three animals treated. All non-polarised cells (Fig. 51) possessed granule distributions similar to those previously observed during luteal phase. The majority of cells displayed a classically polarised distribution of granules. A smaller proportion were polarised incorrectly. The percentages of irregularly polarised cells in two of the three animals were 35% and 15% respectively. A determination of irregular polarisation in the third animal was impossible due to poor fixation which resulted in the destruction of the vascular ultrastructure.

The morphological appearance of the irregular polarisation varied. Granules were located in one half of the cytoplasm as if polarising towards the adjacent cell (Fig. 52). In other instances, granules remained in the aspect of the cell furthest removed from the adjacent sinusoid (Fig. 53).

All cells, regardless of their polarisation status, possessed numerous light dense bodies (Fig. 54). The size and number of these LHβ positive organelles were greatly increased when compared to the natural cycle. In the two animals with well fixed ultrastructure, the mean number of light dense bodies per granule profile was 3.65±0.4 and 6.1±1.2 respectively.

Figure 51. Transmission electron micrograph showing a non-polarised immunoidentified gonadotroph (G) following treatment with oestradiol benzoate. The secretory granules were distributed throughout the entire cytoplasm. Light dense bodies (↟) were numerous, intensely immunoreactive for LHβ subunit with no apparent bias in their distribution towards the nearest sinusoid (S). Magnification = 11500X. Scale bar = 1.7mm.

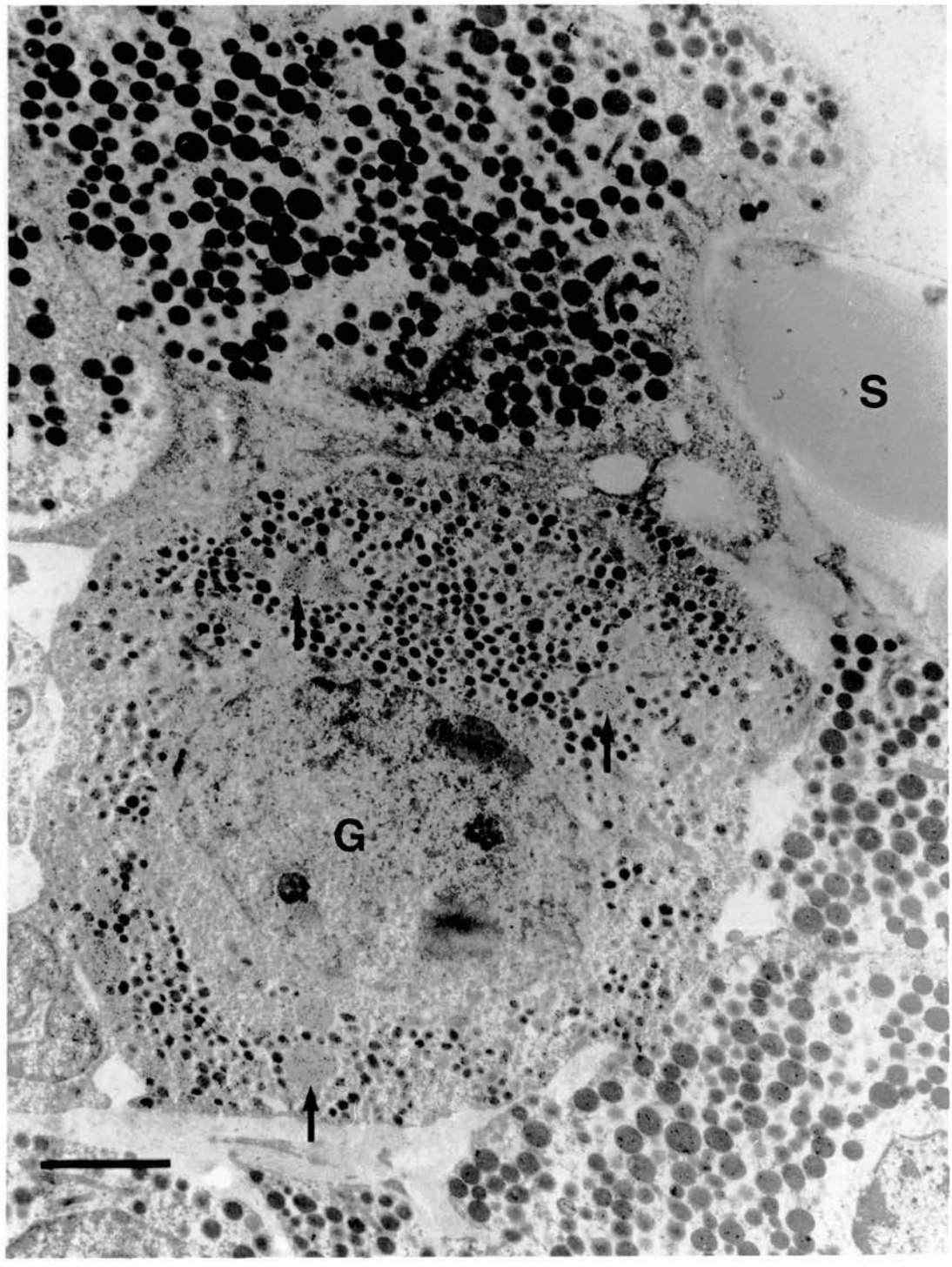

S
G

Figure 52. Transmission electron micrograph showing granule distribution in an immunoidentified gonadotroph (G) after treatment with oestradiol benzoate. In this instance, the granule body (g) was directed towards the adjacent cell and not towards the collagen (c) which had been observed surrounding blood vessels (Fig. 37). Magnification = 9100X. Scale bar = 2.1mm.

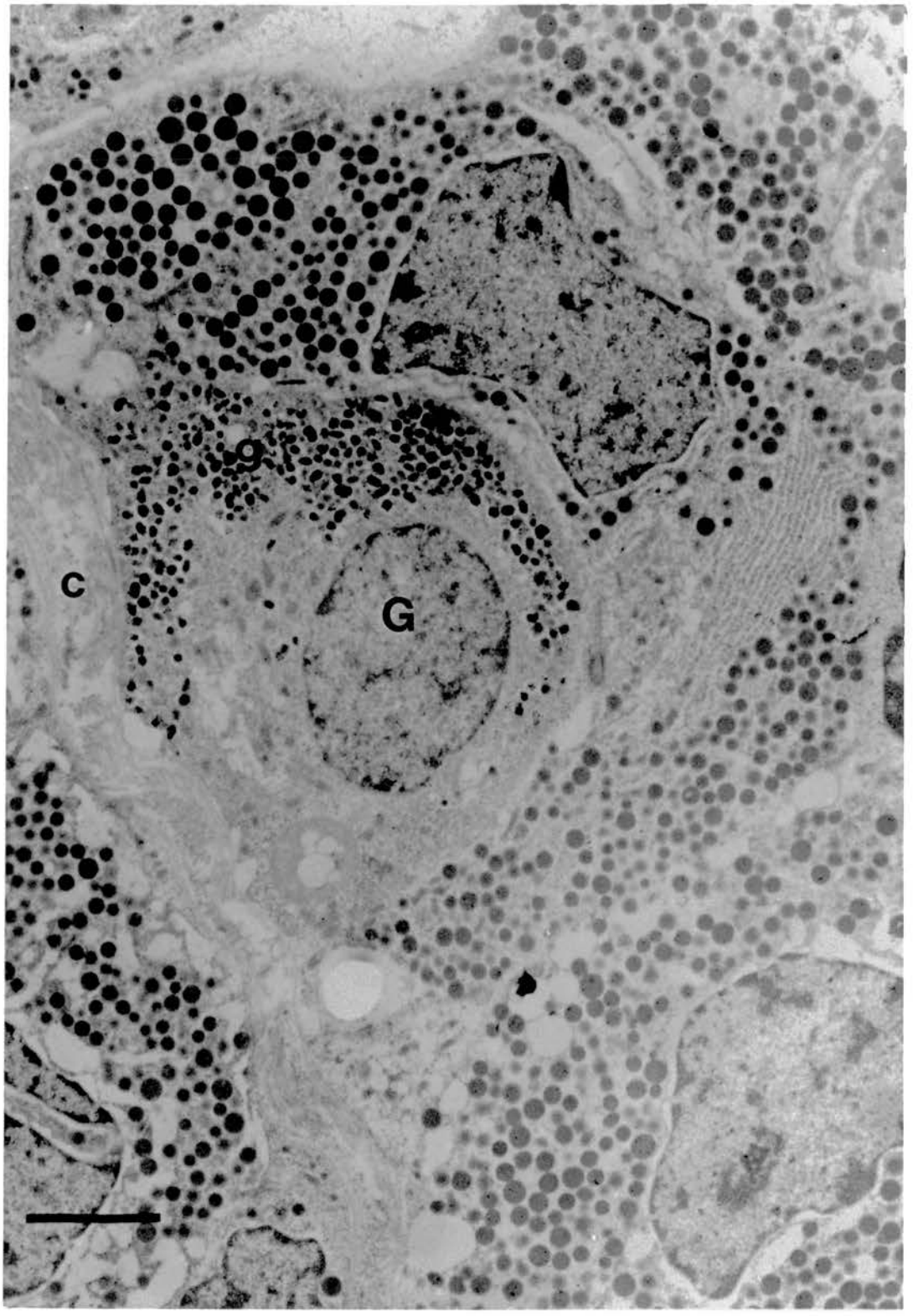

C
G

Figure 53. Transmission electron micrograph showing granule distribution in an immunoidentified gonadotroph (G) after treatment with oestradiol benzoate. The granule body (b) was polarised towards the end of the cell furthest from the adjoining sinusoid (S). Magnification = 8900X. Scale bar = 2.2mm.

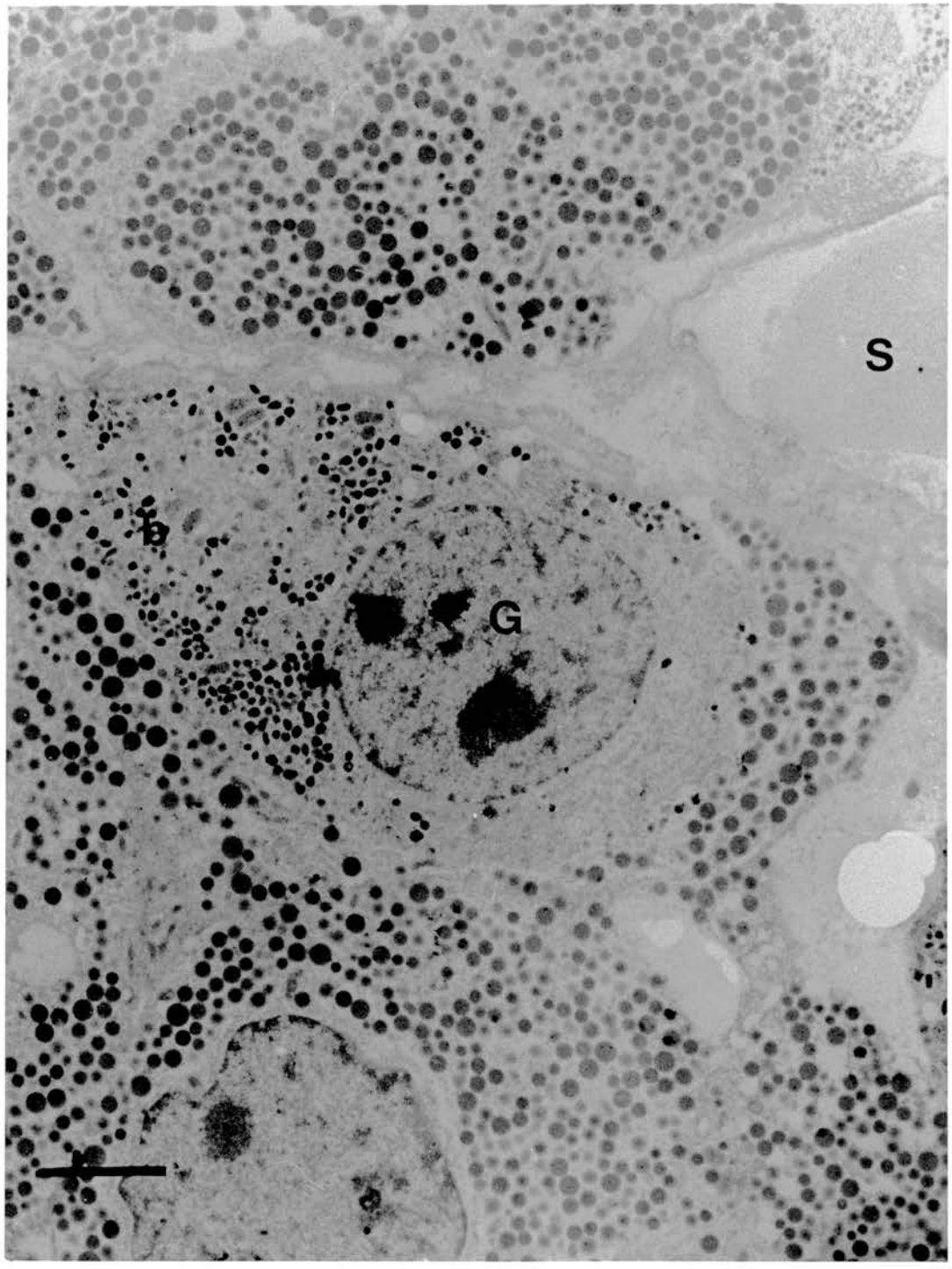
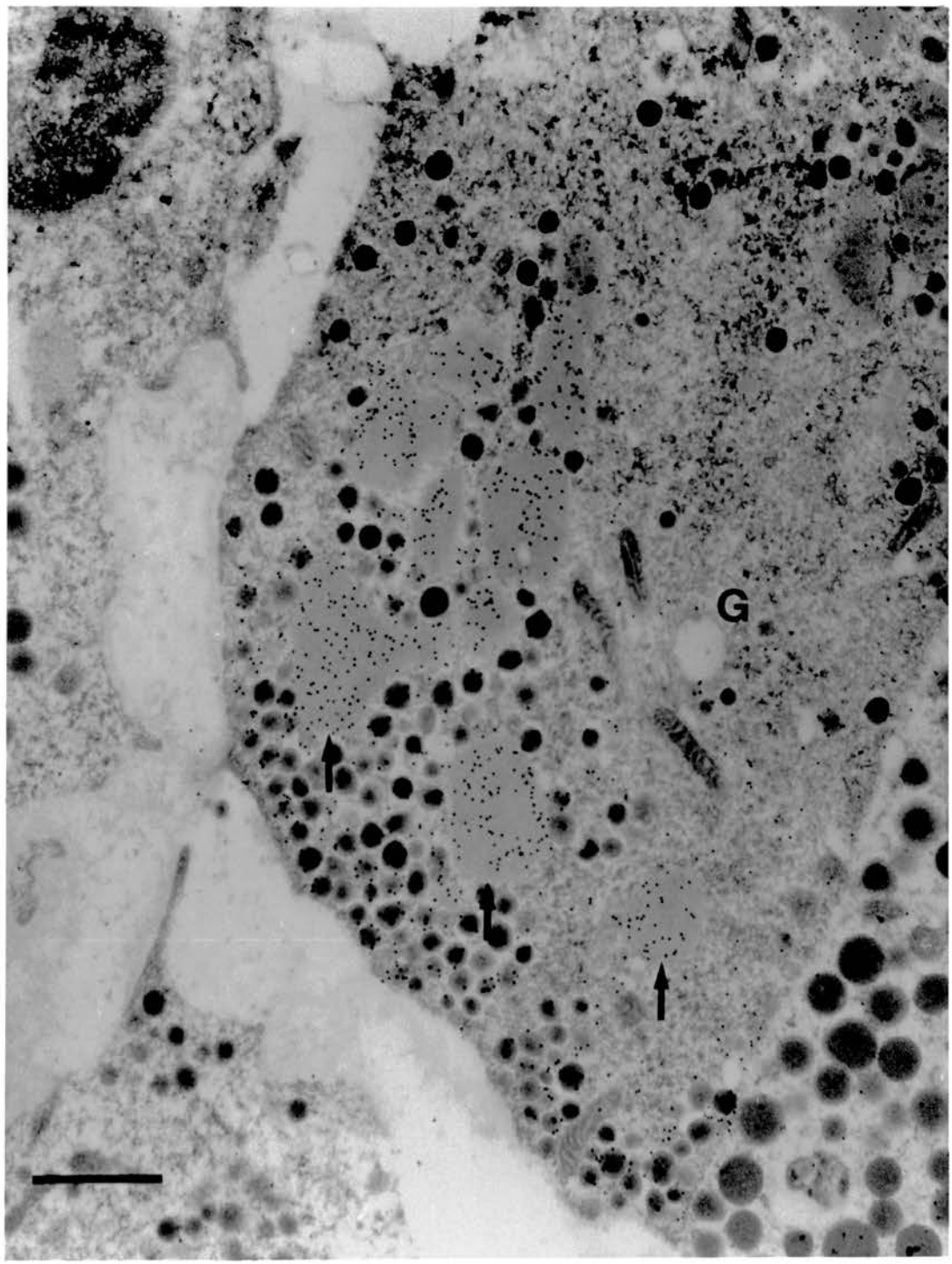

S
b
G

Figure 54. Treatment with oestradiol benzoate induced the appearance of large numbers of light dense bodies (↟). This transmission electron micrograph illustrates light dense bodies in a polarised gonadotroph (G). These LHβ immunopositive, predominantly ovoid organelles did not appear to be polarised to the same degree as the granule body (see also Fig. 37). Magnification = 18500X. Scale bar = 1.1mm.


G

4.6.3. Discussion

Bolus administration of supraphysiological levels of oestradiol lead to the polarisation of the majority of gonadotrophs in luteal phase ewes. Although the percentage of polarised cells was comparable with animals approaching the LH surge during the normal cycle, the direction of the polarisation, in some instances, was not. If the signal to polarise is of vascular origin, it is unlikely that the cells will misdirect their granule stores. Perhaps the supraphysiological levels of oestradiol partially disrupt the cytoskeletal networks which must be required to transport the granule body around the cell. This could result in the emptying of one side of the cell whilst the remaining LH stores, which may have been affected by the cytoskeletal failure and hence incorrectly located for exocytosis, would be unreleasable.

The nature of the light dense bodies is still unclear. Although having a similar appearance to those observed in the rat, their regulation is fundamentally different. In the rat, the organelle was present in more cells and in increased numbers within some cells 92 days postovariectomy (Garner and Blake 1981). This indicates an increase in the absence of oestradiol, where as the present study induced an increase in response to oestradiol administration.

The membrane-bound LHβ-immunopositive organelles did not appear in analysable numbers within granule profiles present in the natural oestrus cycle. Their upregulation in response to oestradiol may be related to an increased rate of synthesis of LH. Alternatively it may be a response to a down-regulation of a protein which is essential for granule condensation and budding from the Golgi, or the maturation of dispersed nascent granules. In the rat, it has been suggested that these bodies are lysosomes (Garner and Blake 1981). The nature and possible functions of light dense bodies will be further discussed in Chapter 7.

4.6.4. Comparison between cell morphologies *in vivo* and *in vitro*

The highly directional nature of the polarisation of the granule body in the ovine gonadotroph suggests that the process may be dependent on the presence of a functional vascular system. The avascular environment of dispersed cell culture, the disruption of cell-cell interactions and the lack of orientation with the basement membrane may all contribute to LH release by non-physiological means. In seminiferous tubules, the basement membrane is responsible for the maintenance of the polarity of the Sertoli

cells (Dym 1994 review). The culture of mammary alveolar cells in agar and treated with laminin allows the cells to form acini and secrete milk into the acinar lumen (Streuli 1993). Thus in other body tissues, the exact composition of the intercellular matrix is pivotal to the orientation of the cell.

4.6.5. Materials and Methods

Cells which had been dispersed with collagenase and cultured for 48h were trypsinized off culture wells and fixed in suspension with 3% glutaraldehyde. The suspension was spun at 500rpm for 2 mins to pellet the cells, the relatively slow speed ensuring the maintenance of good ultrastructure. Following-post fixation in 1% osmium tetroxide, the specimens were sectioned and viewed under the transmission electron microscope.

4.6.6. Results and Conclusion

On rare occasion, pairs of cells structurally identified as lactotrophs (Fig. 55) displayed polarised granule distributions. The granule body was located at the extremity of the cells, at the aspect furthest from the junctional complexes observed at the point of contact (Fig. 56). Similar complexes have been observed in rat pituitary aggregate cultures (Van Der Schueren *et al* 1982). As prolactin is being released from these cultures (due to the removal of the inhibitory dopaminergic tone from the hypothalamus), this is suggestive of a signal from surrounding cell types *in vivo* which directs secretion away and thus towards the nearest blood vessel. Clearly, the signal must be fundamentally different from any which emanates from the endothelial cell of the blood vessel wall.

It has not been determined if this effect occurs in all cell types due to the absence of intercellular contact in the culture examined. The observations do call into question the means by which gonadotrophin secretion is achieved in ovine pituitary cell culture. Although the data demonstrates possible releasing patterns of gonadotrophs, they do not reflect potential paracrine influences from surrounding cells and connective tissues. Thus mechanisms of release and their cellular control may be artifactual.

Figure 55. Transmission electron micrograph showing two structurally identified lactotrophs cultured on a cell culture membrane. The granule bodies (g) were located at the aspect of the cells furthest from the point of contact (↟). Magnification = 8300X. Scale bar = 2.4mm.

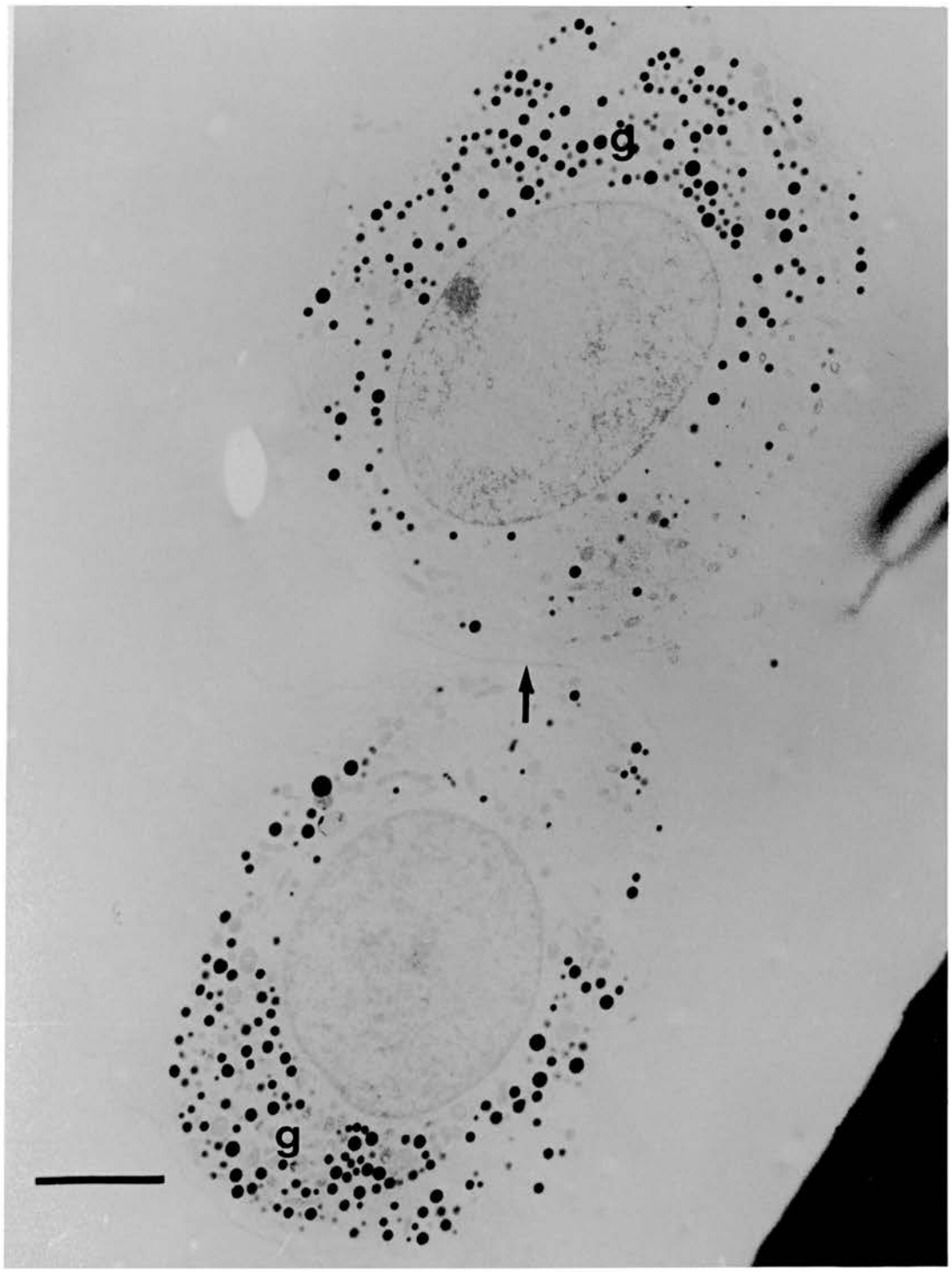
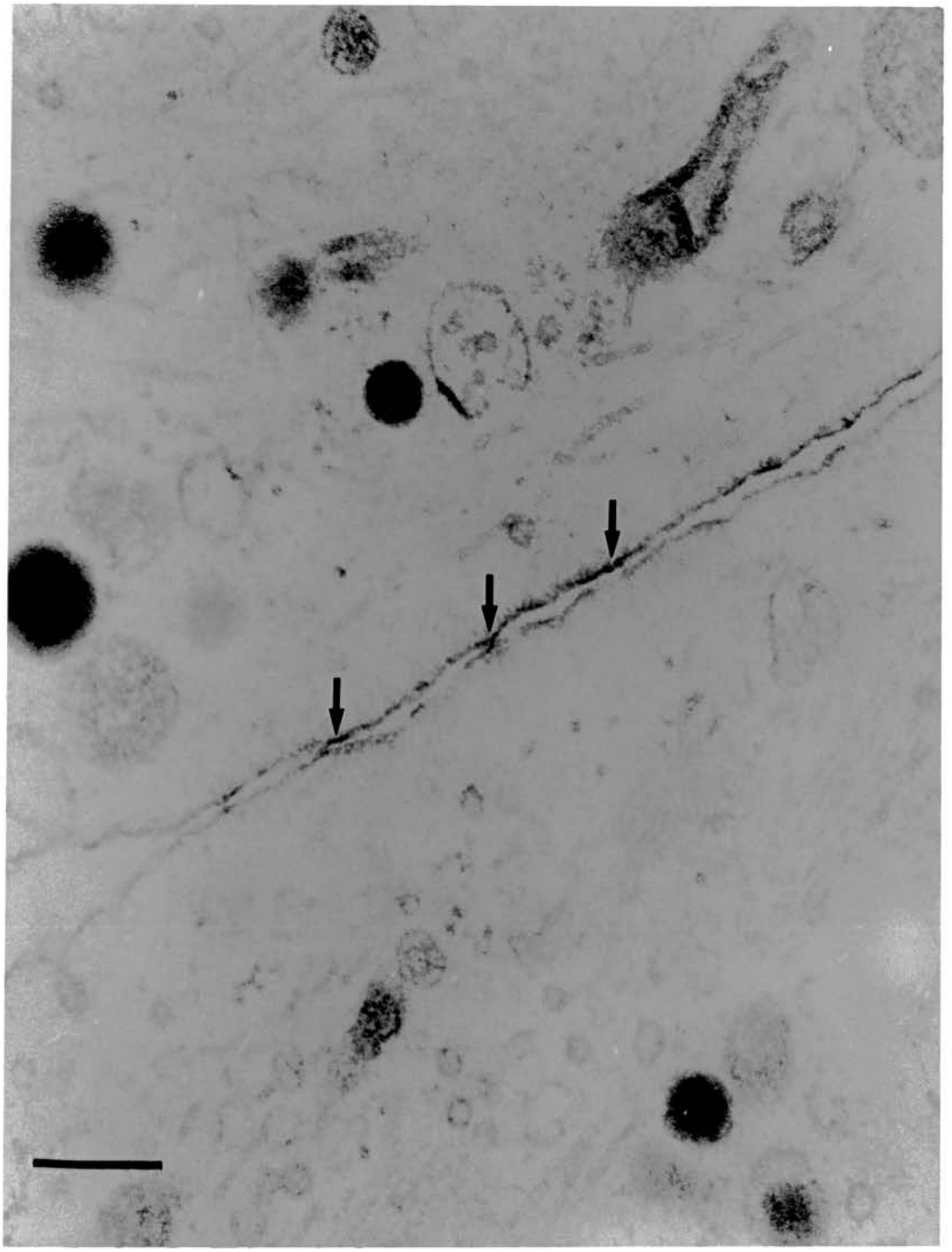

g
g

Figure 56. At the point of contact between the two cells shown in Figure 55, thickened regions were observed on each of the respective cell membranes (↟). At the same points, extracellular deposits filled the intercellular space forming putative functional complexes similar to those described by Van Der Schueren *et al* (1982). Magnification = 89000X. Scale bar = 225nm.



CHAPTER 5

SYNTHESIS AND SECRETION OF LH DURING THE EARLY LUTEAL PHASE

Introduction

The sensitisation of the pituitary gland to GnRH (Clarke and Cummins 1984) and an increasing GnRH pulse frequency and subsequent surge under the control of oestradiol (Clark *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994) lead to the release of the preovulatory LH surge and ovulation. During the preovulatory LH surge, the ovine pituitary gland empties around 80% of its total LH content (Brooks *et al* 1993). Resynthesis of LH is therefore required to allow the animal to complete the next oestrous cycle. The studies detailed in Chapter 4 suggested that the late luteal phase and early follicular phase may be significant periods of LH synthesis during the ovine oestrous cycle. The absence of secretory granules 24h after behavioural oestrus, towards the end of the LH surge, the lowered pituitary LH content and decreased LHβ mRNA abundance measured at this time indicated that hormone synthesis and packaging had not yet increased significantly.

As the patterns of replenishment of LH stores immediately after the preovulatory surge in the sheep have not been investigated previously, a study was undertaken to examine gonadotroph morphology in the period immediately after the LH surge and relate any changes observed to the level of LHβ gene expression.

5.1. Materials and Methods

5.1.1. Animals and Experimental Design

Twenty four Welsh Mountain ewes were randomly assigned to four groups (n=6 per group). Oestrous cycles were synchronised as previously described (**2.2.1.**). Following the intravenous administration of 100μg buserelin (B) to induce an LH surge, 24h after PG before the natural LH surge, the four groups of animals were killed at B+24h, 48h, 72h and 96h respectively. Blood samples were taken at 2 hourly intervals throughout the study and assayed for LH to determine the endocrine status of the animal. In

addition, blood samples were taken every 10mins in the 6 hours immediately prior to death to investigate the pulsatile nature of the LH secretion in each animal. The LH radioimmunoassay sensitivity was 0.3ng/ml. The intra- and inter-assay coefficients of variation were 11.2% and 11.4% respectively.

5.1.2. Electron Microscopy and Stereology

At death, pituitaries were removed, slices from the adenohypophyseal mid-region fixed and prepared for transmission electron microscopy as previously described (**2.2.6.2.**(a)). LHβ and α subunits were localised on ultrathin sections using the immunogold method (**2.2.6.3.**(b)). Stereological assessment of granule size was made from four animals randomly selected from each group. Systematic random sampling was used to locate 13 cell profiles per animal. Cells were photographed and granule profiles analysed by the Schwartz-Saltykov diameter analysis (**2.2.6.4.**).

5.1.3. Analysis of Pituitary Content, mRNA Abundance and GnRH Binding

Following removal of a 1mm thick tissue slice for immunocytochemistry, tissue homogenates were prepared by homogenising tissue in cold 0.3M sucrose/1mM EDTA/tris HCl, pH 7.4 medium (SET) using 1ml of medium/ 100mg tissue, and used to determine the pituitary LH content (assay sensitivity = 2ng/ml, intra assay coefficient of variation = 11.6% and GnRH-binding activity (**2.2.4.**). The remaining pituitary tissue was processed and the total RNA subjected to Northern analysis for LHβ mRNA as previously described (**2.2.7.**).

5.2 Results

5.2.1. LH Levels, mRNA Abundance and GnRH Binding

In all animals, the assay of plasma samples confirmed the production of an LH surge (Fig. 57). The duration of the surge was approximately 10 hours. At time of death, plasma LH concentrations were 0.96±0.10ng/ml at B+24h, and 1.19±0.13ng/ml at B+48h. At B+72h, the concentration rose to 1.42±0.32ng/ml before arriving at a final value of 1.39±0.28ng/ml at B+96h. These concentrations did not differ significantly.

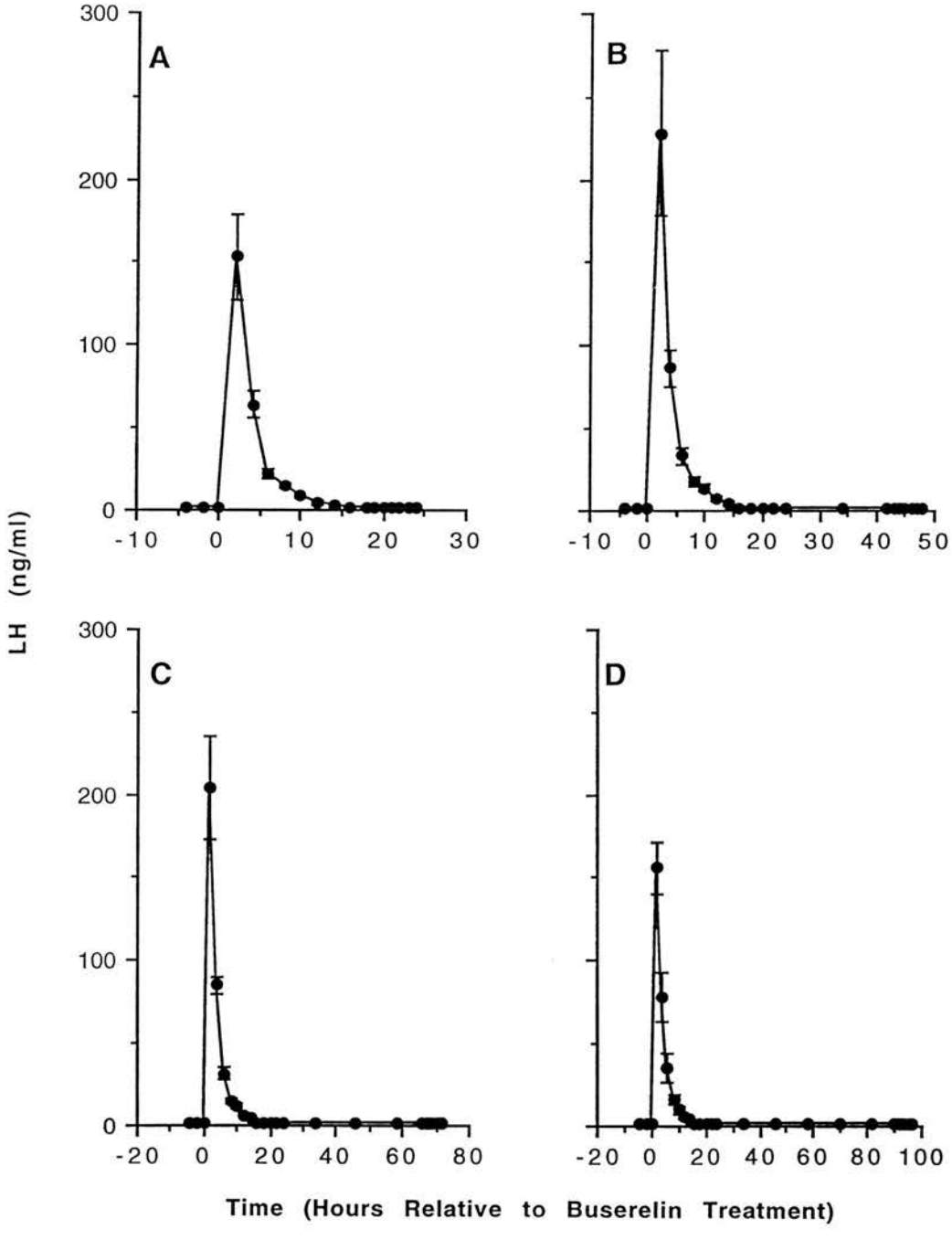


Figure 57. Changes in the plasma concentration of LH following the induction of an LH surge with the GnRH agonist buserelin, 36h after prostaglandin-induced luteolysis. Samples were collected every 2h from 2h before to 18h after buserelin and at 1 to 8h intervals until animals were killed at (A) 24h, (B) 48h, 72h (C) and 96h (D) after buserelin. Values are mean ± SEM, n = 6 per group.

Parameters of pulsatile LH secretion are shown in Table 4:

Table 4. LH pulse frequency, pulse amplitude and basal concentrations 24, 48, 72 and 96h after an induced LH surge. Values are given as mean ± SEM and n = 6 in all groups. Pulse frequencies are expressed as pulses/6h to allow direct comparison between these and pulse frequencies in Chapters 4 and 6. * mean pulse frequency composed of one pulse from a single animal.

Hours after induced induced LH Surge	Pulse Frequency (Peaks/6h)	Pulse Amplitude (ng/ml)	Basal LH (ng/ml)
*24	0.17 ± 0.17	0.43	1.03 ± 0.11
48	3.17 ± 0.91	0.98 ± 0.20	0.99 ± 0.14
72	2.83 ± 0.70	1.06 ± 0.22	0.85 ± 0.13
96	3.17 ± 1.19	1.05 ± 0.20	1.12 ± 0.16

Representative LH pulse profiles from individual ewes from each group are shown in Figure 58.

Pituitary LH content increased significantly ($P<0.05$) from 24 to 48h after the buserelin-induced LH surge (Fig. 59a). Although LH content continued increase 72 and 96h, these values did not differ significantly from those at 48h after the induced LH surge. In contrast, the levels of LHβ mRNA displayed no significant differences at the four time points examined (Fig 59b). GnRH-binding (Fig. 59c), increased significantly ($P<0.05$) from 24 to 48h and from 48 to 72h following the induced LH surge. Although binding activity appeared to decrease at B+96h, this did not differ significantly from B+48h or B+96h values.

5.2.2. Ultrastructural Observations

At B+24h, cell profiles were devoid of electron dense granules (Fig. 60). At higher power, immunogold staining revealed the presence of LHβ subunit in the cytoplasm (Fig. 61), although at no time was this immunoreactivity contained within any visible granule structure. The agranular cytoplasm of these cells was immunonegative for α subunit.

Small, punctate, electron dense granules were present at B+48h and were LHβ immunopositive (Fig. 62). Immunoreactivity of the α subunit was detected within the same granule (Fig. 63) and located mainly in the granule periphery. The granules were

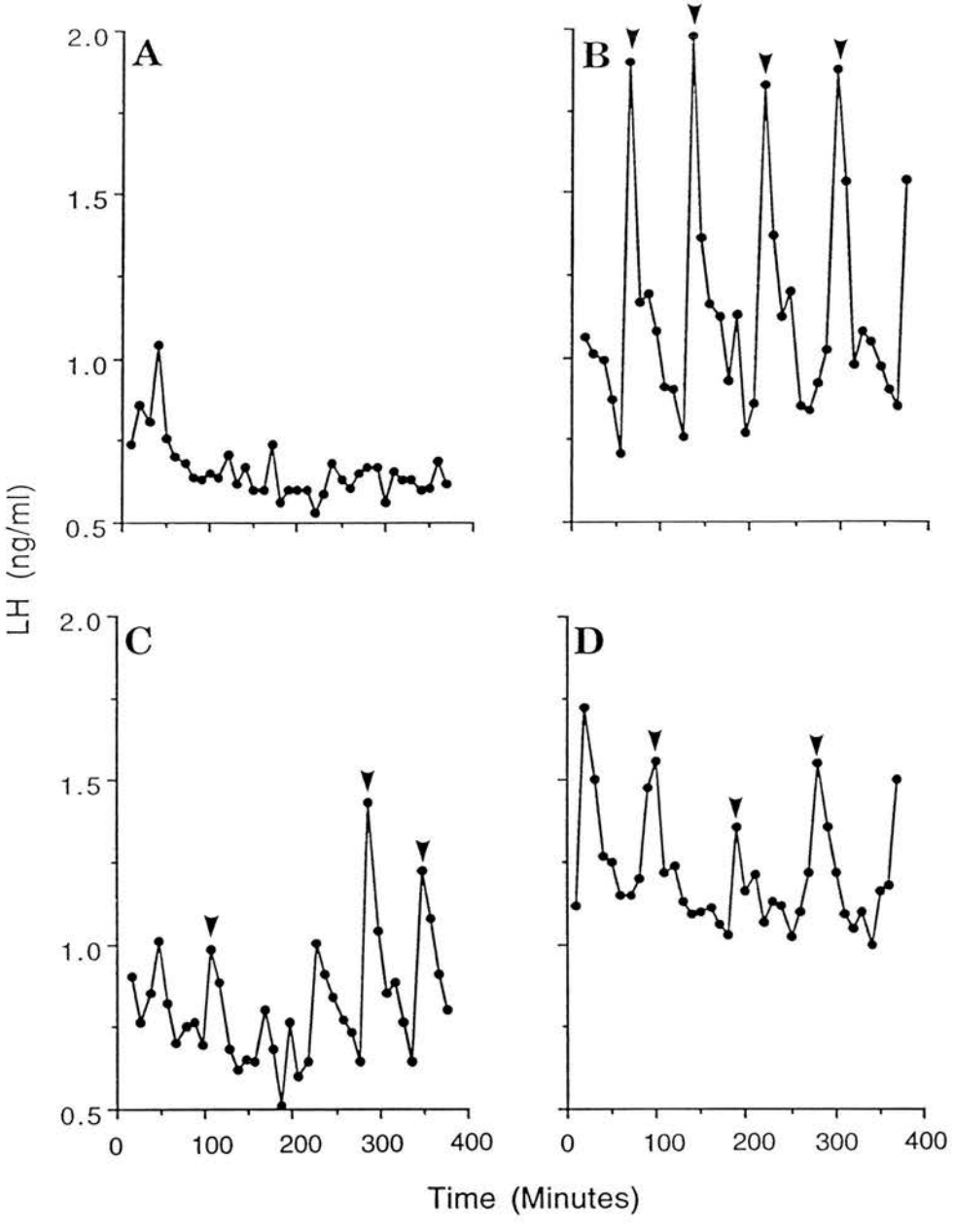


Figure 58. Representative profiles of pulsatile LH secretion from individual ewes in the 6h period immediately prior to death at (A) 24h, (B) 48h, 72h (C) and 96h (D) after the induction of an LH surge with the GnRH agonist buserelin. Arrowheads indicate significant LH pulses.

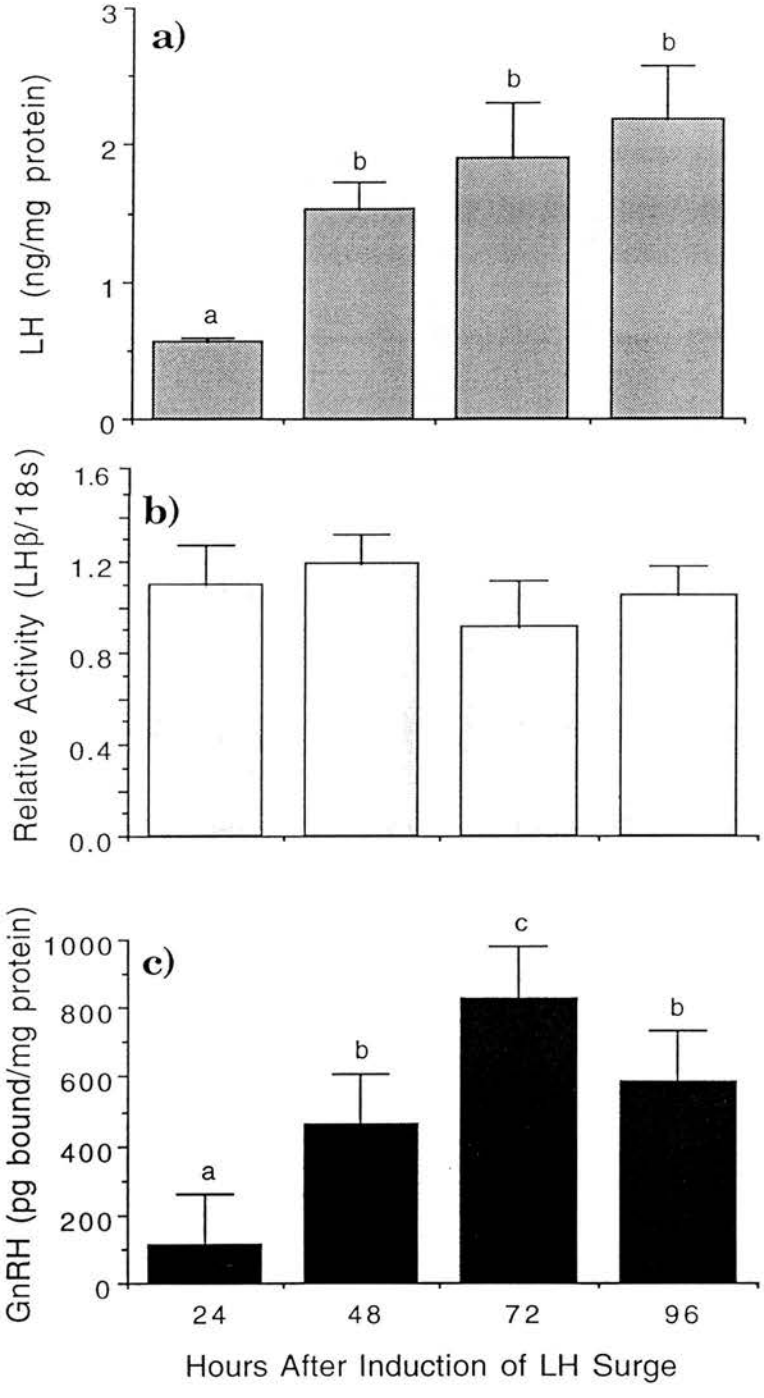


Figure 59. Changes in a) pituitary LH content, b) LHβ mRNA abundance expressed relative to 18S ribosomal mRNA and c) levels of GnRH binding in ewes (n=6 per group) killed at 24h, 48h, 72h and 96h after the induction of an LH surge. Data was analysed by one-way ANOVA. Values are given as mean ± SEM and different letters indicate a significant ($P<0.05$) difference.

Figure 60. Transmission electron micrograph showing an immunoidentified gonadotroph (G) and adjoining sinusoid (S) 24h after the induction of an LH surge by the GnRH agonist buserelin. The gonadotroph cytoplasm was completely devoid of secretory granules. Magnification = 12000X. Scale bar = 1.7μm.

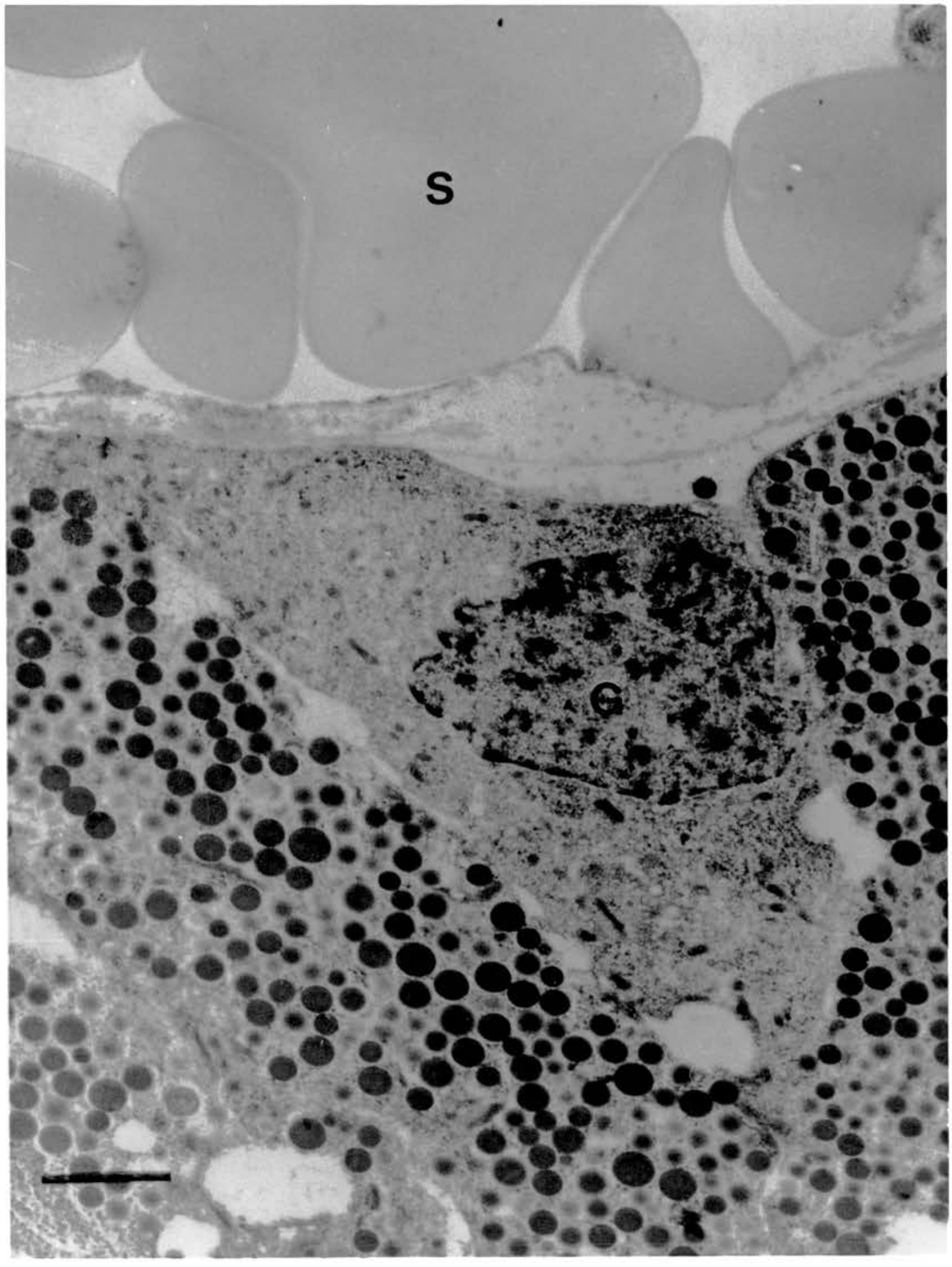

S
C

Figure 61. At higher magnification in the buserelin plus 24h group, distinct areas of LHβ immunoreactivity (↟) were observed in the gonadotroph cytoplasm without the presence of granule structure. This staining pattern was not observed over the gonadotroph nuclear profile (N) or in the cytoplasm of other pituitary cell types. Magnification = 84500X. Scale bar = 235nm.

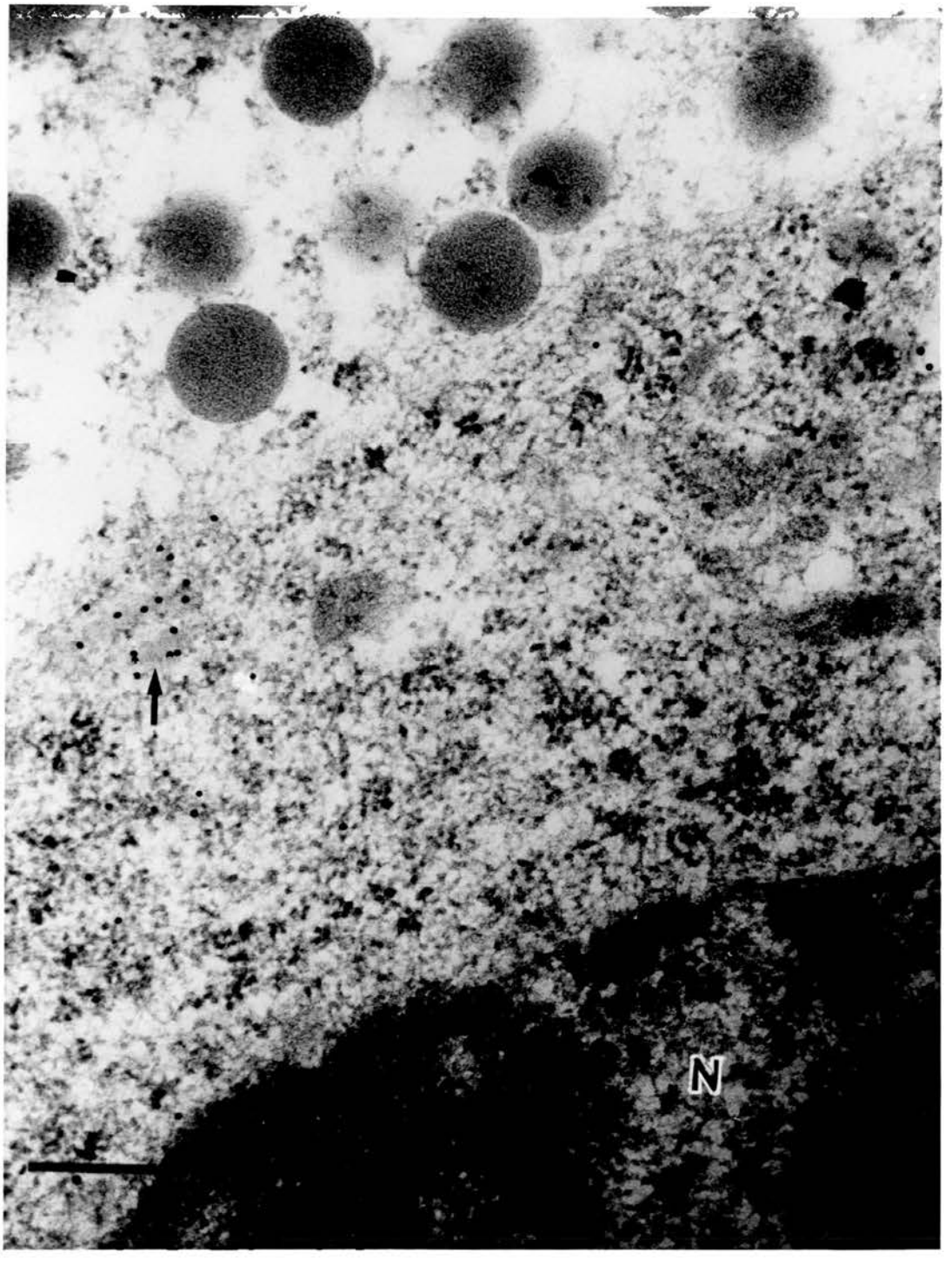

N

Figure 62. This transmission electron micrograph shows an immunoidentified gonadotroph (G) 48h after GnRH agonist treatment. A few small LHβ positive secretory granules (↟) were present in the aspect of the cell nearest the sinusoid (S). The cell contained numerous mitochondria (M). Magnification = 10000X. Scale bar = 2.0μm.

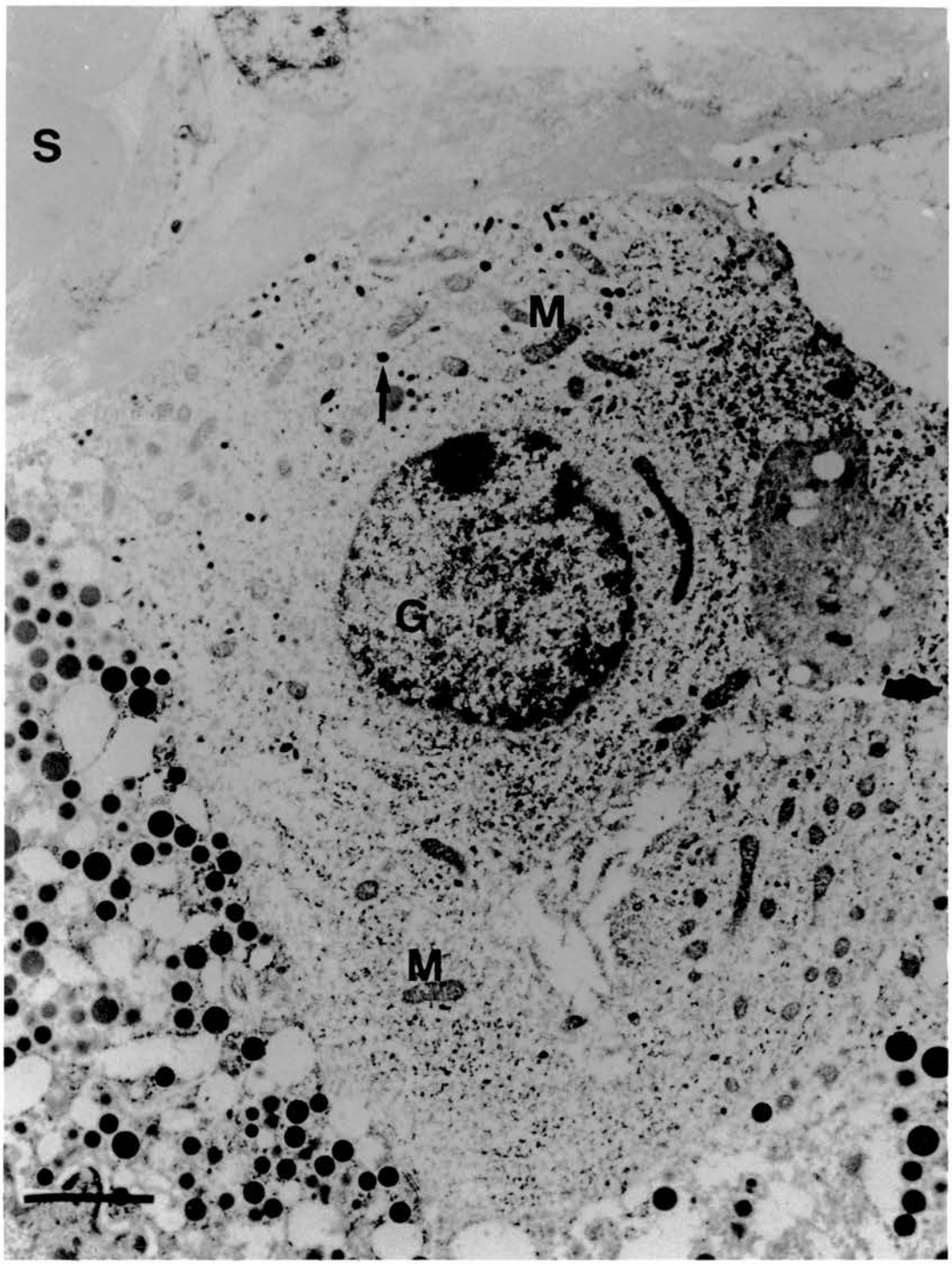
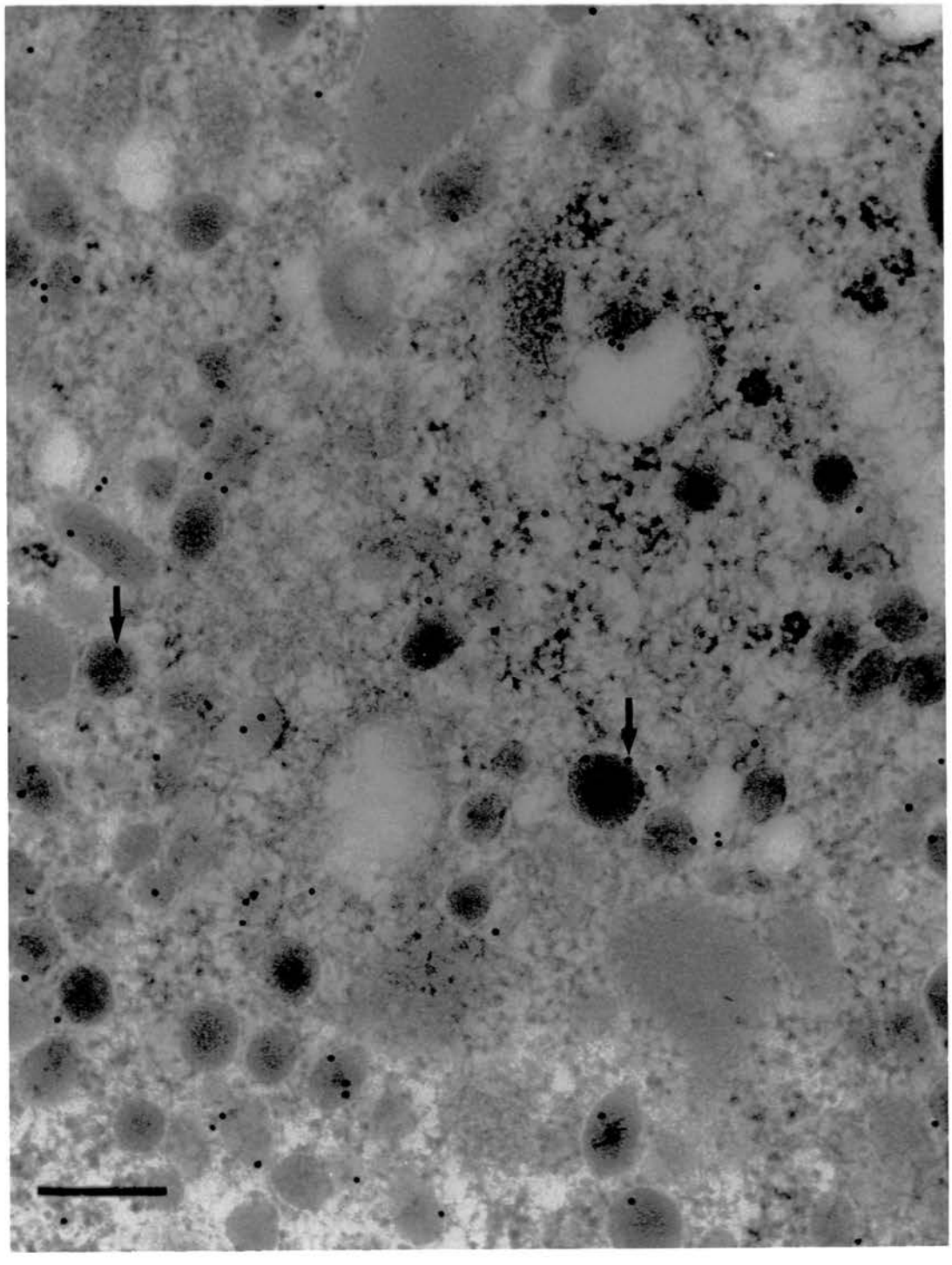

S
M
G
M

Figure 63. The gonadotrophin α subunit (↟) was identified in secretory granules 48h after GnRH agonist treatment with 15nm gold particles using the immunogold technique. The α subunit was located preferentially towards the periphery of the secretory granule. No convincing evidence was found for the presence of α subunit in the cytoplasm. Magnification = 90500X. Scale bar = 220nm.



located, predominantly, at the aspect of the cell juxtaposed to the vascular system. By B+72h, the size of the intracellular granule body increased with the increase in numbers emanating from the side of the cell nearest the sinusoid (Fig 64). In the gonadotrophs of animals from the B+96h group, replenishment of the LHβ immunopositive granule population had progressed to the extent that the morphology of some cells approached that of a classically non-polarised cell (Fig. 65) whilst others appeared polarised.

At the time points investigated, no changes were observed in the cortical cytoskeleton. The increase in the prominence of profiles of RER observed at B+24h was attributed to the absence of secretory granules unmasking the organelles' structure.

5.2.3. Stereology

The Schwartz-Saltykov analysis demonstrated the existence of a class of granules of approximately 60-90nm in diameter (Fig. 66). This class, with one exception (Fig. 66, a, 1) was present at B+48h and B+72h but absent at B+96h. By 96h after buserelin administration, granule size distributions approached those measured in animals during a natural luteal phase. However, the modal class (0.4) was smaller than the fully stocked non-polarised gonadotroph (0.5). The content of the 0.5 and above classes was still reduced when compared to luteal values (Chapter 4).

The mean number of granules (Table 5) increased significantly ($P<0.05$) from 48 to 96h after the induction of an LH surge. The value of $11.61 \pm 0.98 \times 10^9$ granules/mm^3 cytoplasm was approximately half the number calculated in non-polarised cells (Chapter 4). Although the mean cytoplasmic area observed per gonadotroph did not differ significantly between individual ewes at B+48h, significant ($P<0.05$) differences in cytoplasmic area did exist within the B+72h and B+96h groups.

5.3. Discussion

Stereological analysis has demonstrated that the replenishment of the intracellular stores of LH occurred by synthesising granules containing LH in a size-dependent order. The smallest granules were present 48h after the induction of an LH surge. By 96h after induction, the larger granule classes became more prevalent, as cell morphology approached that of cells with a non-polarised granule distribution. Granule profiles were located initially in the vicinity of the plasmalemma nearest the vascular bed.

Figure 64. By 72h after GnRH agonist treatment, gonadotrophs (G) contained more secretory granules (↟). Granules were less dispersed than at 48h after GnRH agonist treatment with the granule body located in closer proximity to the cell membrane. Magnification = 13500X. Scale bar = 1.5μm.

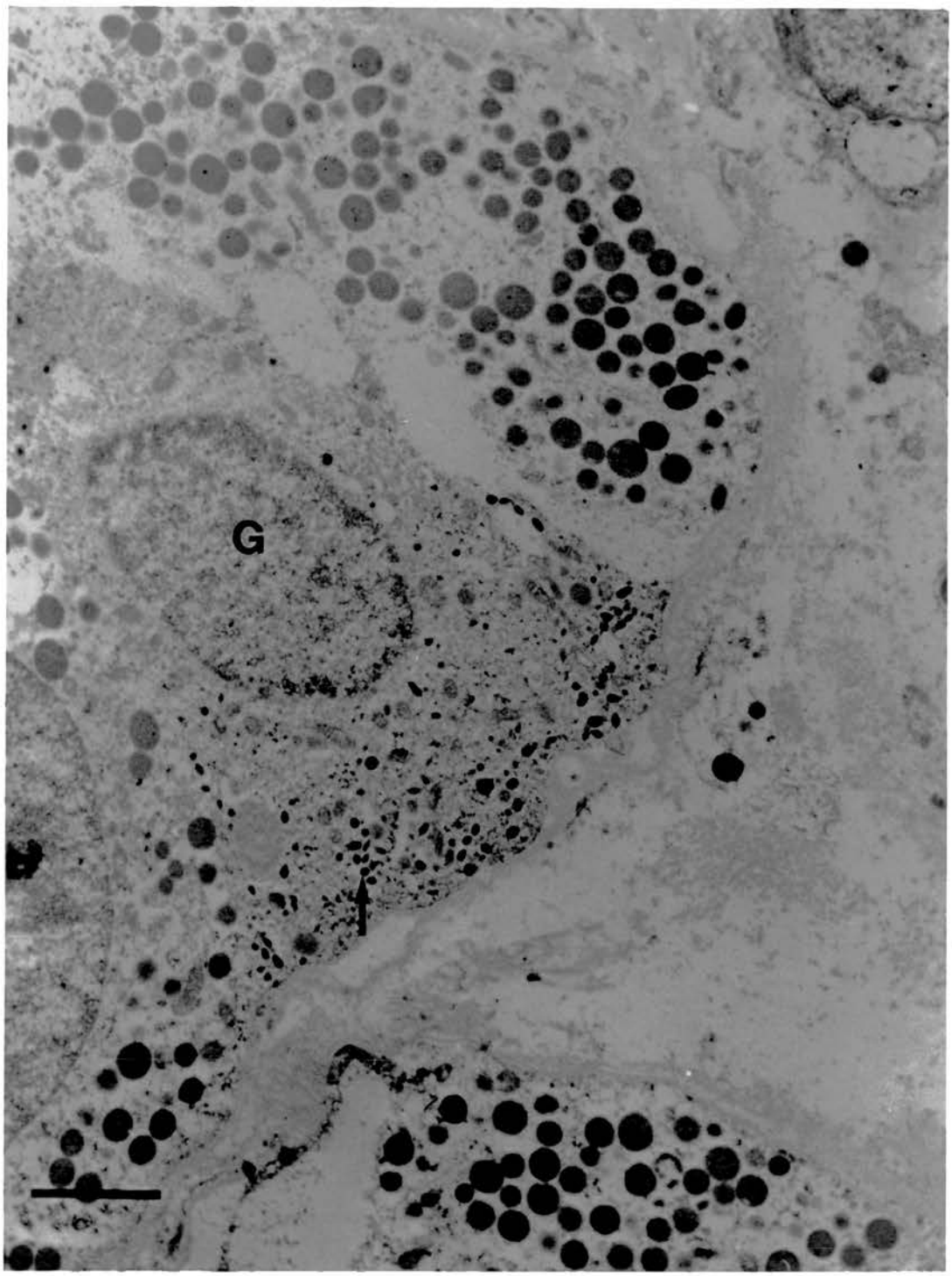
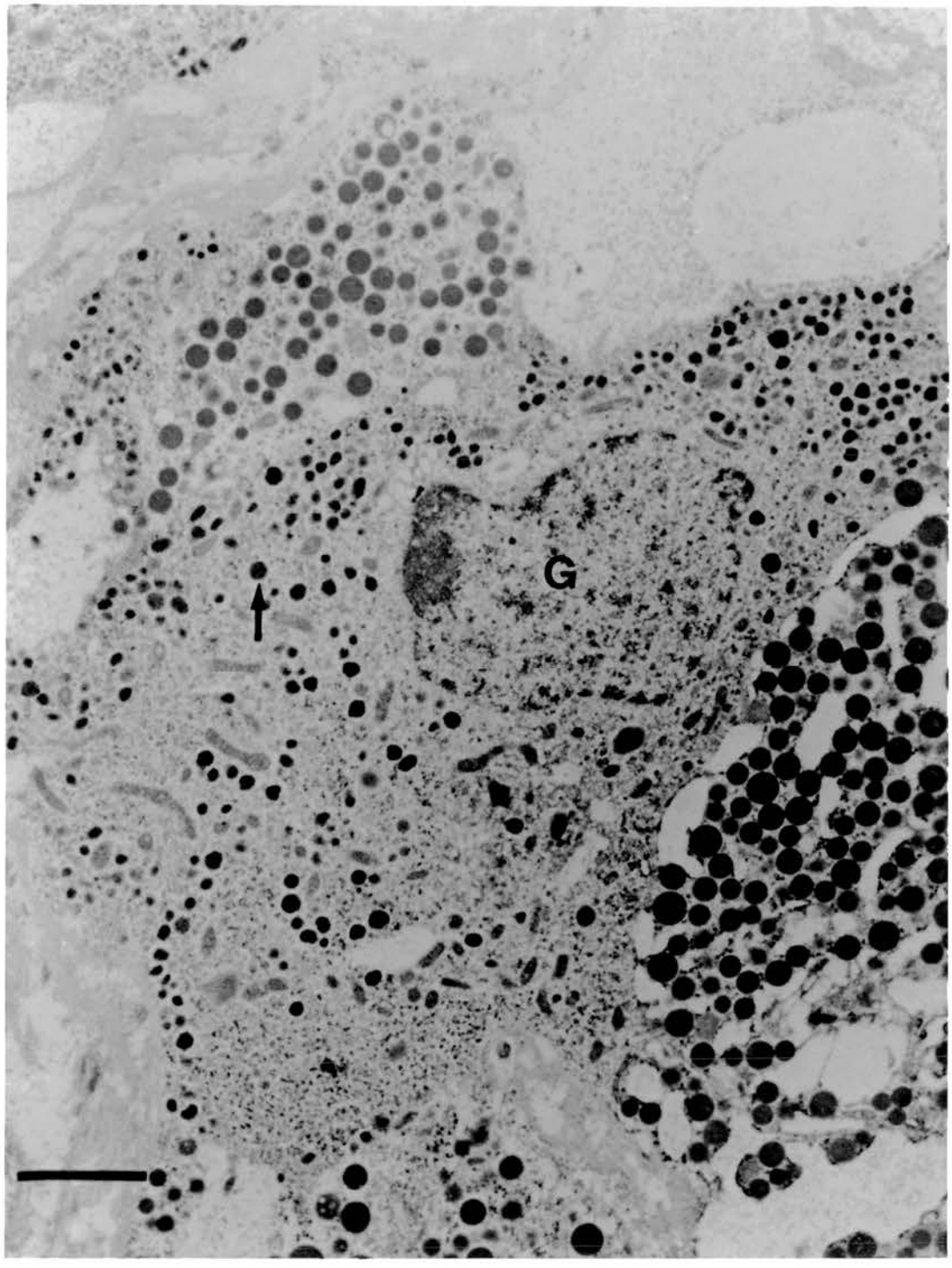

G

Figure 65. This transmission electron micrograph shows a gonadotroph (G) 96h after treatment with a GnRH agonist. The LHβ positive secretory granule profiles (↟) were notably larger than at 48h and 72h after the buserelin induced LH surge. Profiles were also more numerous and distributed throughout the entire cytoplasm giving the cell a non-polarised appearance. Magnification = 11500X. Scale bar = 1.7μm.


G

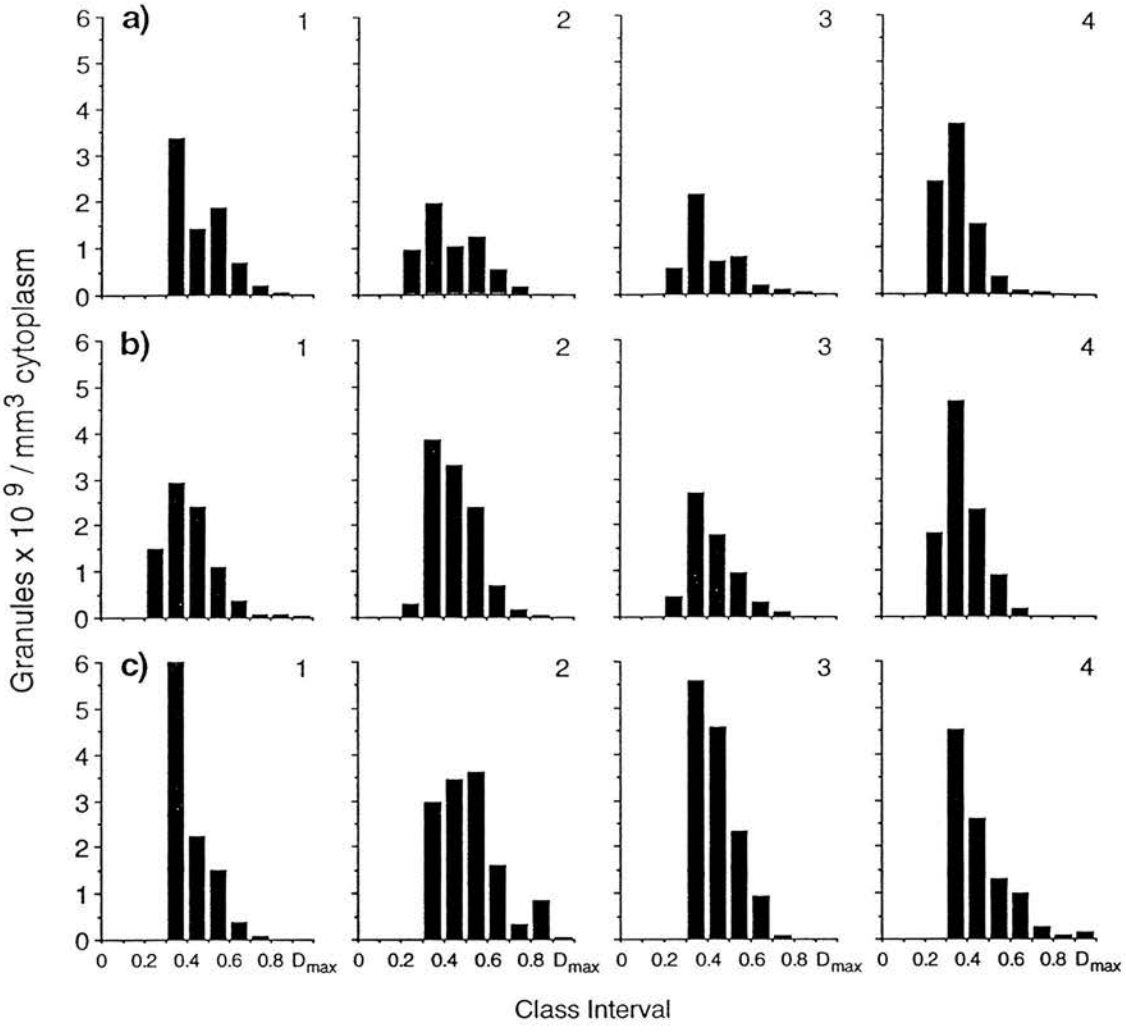


Figure 66. Comparison of Schwartz-Saltykov diameter analysis distributions showing numbers of granules $(10^9)/mm^3$ of cytoplasm in each diameter class interval from four animals (numbered 1 to 4) a) 48h, b) 72h and c) 96h after the induction of an LH surge. Class intervals are 10% decrements of the maximum granule diameter (D_{max}) which was 300nm.

Table 5. Summary of data from individual animals from 48, 72 and 96h after an induced LH surge from the Schwartz-Saltykov diameter analysis. Stage of cycle numbers correspond Schwartz-Saltykov plots in Figure 66. Total cytoplasmic concentration of granules from all size classes and gonadotroph cytoplasmic area (mean ± SEM, n = 13) are shown. Differences in cytoplasmic areas and mean granule number were analysed by one-way ANOVA and different letters indicate a significant ($P<0.05$) difference.

Hours after induced LH Surge	**Total Number of Granules ($x10^9/mm^3$ cytoplasm)**	**Gonadotroph Cytoplasmic Area (mm^2)**	**Mean Number of Granules ($10^9/mm^3$ cytoplasm)**
48			
1	7.50	65.54 ± 28.22^{a}	
2	5.60	$54.28 \pm 9.62^{a,d}$	
3	3.47	$49.90 \pm 4.65^{a,e}$	6.15 ± 1.04^{a}
4	8.05	49.72 ± 5.17^{a}	
72			
1	8.43	63.16 ± 5.12^{a}	
2	10.61	$42.12 \pm 17^{b,d,e}$	
3	6.21	$45.57 \pm 6.85^{b,d,e}$	8.77 ± 0.97^{b}
4	9.83	32.67 ± 4.41^{b}	
96			
1	10.16	$36.91 \pm 3.95^{b,e}$	
2	12.72	50.14 ± 5.29^{a}	
3	13.80	53.31 ± 6.24^{a}	11.61 ± 0.98^{b}
4	9.77	$47.18 \pm 6.28^{b,e}$	

Throughout the period of resynthesis under investigation, the levels of LHβ mRNA did not change significantly.

The decreased pituitary LH content and mean number of granules/mm^3 cytoplasm 96h after the induction of an LH surge compared to the luteal phase pituitary and non-polarised gonadotrophs, suggests that further gonadotrophin synthesis and packaging are required to fully replenish the LH stores. Presumably, this process would be completed before day 12 of the luteal phase (see Chapter 4) after which LHβ mRNA abundance and pituitary LH content begin to decrease.

The biological significance of the synthesis of the smallest granule sizes before large ones is unclear. In this study, the gonadotrophs were able to release LH in a pulsatile fashion 48h after the end of the induced LH surge. The apparent synthesis of granules in the region of the cell from which they are most efficiently released is biologically advantageous. It may be that small granules are more readily able to pass through the filamentous actin cortex and thus achieve exocytosis. The cortex may still be in a partial disassembled state following the probable scinderin-mediated (Trifaro 1992, 1993) perturbation of its structure during the preceeding LH surge. This mechanism would allow release, although the pituitary may be relatively unresponsive to GnRH at this time due to the GnRH-binding activity and GnRH receptor mRNA being decreased significantly following the LH surge (Brooks *et al* 1993). The passage of the small granules out of the cell would therefore allow the re-establishment of a basal LH output. This adaptation may also facilitate a faster return of pulsatile LH secretion due to an increased ease of exocytosis despite the relative lack of sensitivity to any GnRH signal. The initial appearance of granules in the region of the cell subjacent to the vascular system, leading to a type of polarised morphology, suggests that the *de novo* synthesis of the granules occurs at a favourable site for exocytosis. This would allow the cells to respond to a secretory stimulus without the requirement to translocate the granule body to the region of the cell associated with exocytosis. Apart from the more rapid response that this would yield, it may be biochemically more efficient thus allowing a greater proportion of the resources to be channelled into synthesis of LH and associated proteins of the cells necessary for granule production. The previous studies in this thesis have demonstrated that polarised morphologies are present at all stages of the ovine oestrous cycle. It is notable that, in almost every cell observed granules appeared, in the first instance, nearest the juxtaposed sinusoid. Two broad theories may be proposed as to the cellular origin of LH secretion immediately after the LH surge:

1. As most cells are "polarised", they will all respond at a low level to any incident GnRH stimulus. As synthesis progresses in the luteal phase, the majority of cells may decrease their response to GnRH but continue hormone synthesis thus becoming non polarised and therefore acting as the storage cell for the majority of the intracellular LH stores. This may imply that the newly formed non polarised cell would then down regulate its GnRH receptor complement in order to become non-responsive. The small proportion of cells which remained polarised, may upregulate their GnRH receptors in order to respond more efficiently to the secretory signal.

2. Only a sub-population of gonadotrophs will respond to the secretory stimulus. This may be the cell cohort which is permanently polarised and therefore constantly synthesising and exocytosing. It would seem that GnRH receptors would be required on the remaining gonadotrophs in order to facilitate LH production but in this hypothesis, a bar on exocytosis would be required in order for the storage pattern to become established.

Due to the observation that almost all gonadotrophs possess a polarised granule distribution post-LH surge, hypothesis 1. appears to be the more tenable. It is difficult to justify the synthesis of all granules at the vascular aspect of the cell if it is not to facilitate release. Equally, preventing the exocytotic stimulus which occurs as a consequence of the action of GnRH on its receptor would appear to be biologically inefficient.

Synthesis of granules in the extremity of the cell may be achieved by the specific targeting of the LHβ mRNA to that region of the cytoplasm. It has now been established that different mRNAs can be localised to distinct areas of the cell. The mRNA species which encode the cytoskeletal proteins α–actin, β-actin, vimentin and tubulin are localised subcellularly in the vicinity of the polymerisation sites of the respective protein products (Lawerence and Singer 1986; Taneja and Singer 1990). The targeting signal for the localisation event has been demonstrated to lie in the 3' untranslated region of α and β actin mRNA species (Kislauskis *et al* 1993). The receptors which identify these signals have not yet been defined. Microtubules and microfilaments have been implicated. This mechanism is therefore attractive as an organised cytoskeletal structure may be present in the polarised gonadotroph to allow granule movement and exocytosis. Targeting β actin mRNA utilises the microfilament network exclusively (Sundell and Singer 1991) whilst a combination of microfilaments and microtubules are required in the subcellular management of *Vg1* mRNAs (Yisraeli

et al 1990). It may be that mRNA species arrive at specific sites on the RER membrane by way of RNA targeting motifs or events requiring the nascent peptide chain (Okita *et al* 1994). The four methods outlined include asymmetrical transcript export from the nucleus; the use of a targeting signal motif in the 3' untranslated region; the translation of a particular mRNA may generate a nascent polypeptide which is transported and inserted into a region of the RER by a specific signal recognition particle (SRP); immunoglobulin heavy chain binding protein ' (BiP), one of a family of luminal binding proteins, may increase the concentration of prolamine mRNAs as the folding kinetics of the luminal product favour retention of the transcript on the ER membrane in the cells of plant seeds. Of these four, asymmetrical transcript export is unlikely to occur in the gonadotroph as the nucleus does not partition the cell into two distinct compartments - the situation which arises in *Drosophila* (Banerji *et al* 1987, Tepass *et al* 1990).

Thus the mechanisms exist by which the cell can carry out *de novo* protein synthesis at a specific location within the cytoplasm. Indeed, the LHβ mRNA 3' untranslated region (D'Angelo-Bernard *et al* 1990) although relatively short, may contain the actual targeting signal. The tracking of a truncated form of LHβ mRNA, and comparison with a full length equivalent, through the cytoplasm in a transfected cell line would determine if this mechanism was operative in the gonadotroph.

The LH content of the pituitary rose throughout the experiment as the gonadotrophin stores were replenished. Although at B+24h, 0.56ng/mg of LH was present, the vast majority of cell profiles were devoid of electron-dense secretory granules. The LH in the pituitary at this time may either be in a non-granular form or contained within cytoplasmic vesicles of low elecron-density, as indicated by LHβ cytoplasmic immunoreactivity (Fig. 61). The appearance of granules at B+48, 72 and 96h was not accompanied by an increased level of LHβ mRNA. There are several factors that may account for this. The total mRNA measurement takes no account of the stability of the species present. Investigations on cultured rat pituitary cells comparing the stabilities of the mRNA species encoding LHβ, FSHβ and the common α subunit have shown that the half life of the mRNA is apparently related to the length of the transcript (Bouamoud *et al* 1992). FSHβ, being the longest mRNA, possessed a half life of 1.0±0.13h. The common α subunit mRNA, of intermediate size, took 6.5±0.25h to decay to 50% of its original abundance whilst LHβ, the shortest transcript, appeared the most stable with a half life of 44±0.5h. Decline in mRNA abundance was associated with a shortening of the transcript length. The cDNA probe used in the current studies on the sheep to determine LHβ mRNA abundance, recognises the first 176 nucleotides from the

5' end of the mRNA. Thus, a slight shortening of the transcript from the 3' end would still allow the detection of the RNA species. The poly A tail at the 3' end together with the poly(A) binding proteins are required for the initiation of protein translation (Grossi de Sa *et al* 1988, Munroe and Jacobson 1990). As well as acting as an initiator for protein translation, the poly(A) poly(A) binding protein complex may, by mediating the assembly of the 60S-40S ribosomal RNA complex (Sachs and Davis 1989), retard RNA cleavage by exonucleases at the 5' end (Brawerman 1993). It is possible that a slightly truncated form of LHβ mRNA, which would not be identified by Northern analysis as a different species, may be present in the period immediately after the LH surge. The unstable nature of this species and the resultant lack of exonuclease protection would lead to a paucity of translated product. The acquisition of 3' initiation functions together with the increased stability related to the increased length of transcript may be responsible for the increased synthesis which manifests in the greater pituitary LH content observed. It has been demonstrated in rats *in vivo* that ovariectomy increases the polyadenylation of LHβ mRNA (Weiss *et al* 1992). In the same study, administration of pulsatile GnRH to dispersed rat pituitary cells resulted in an increase in the length of the LHβ mRNA poly(A) tail . This effect is mediated via cAMP (Ishizaka *et al* 1993). Thus GnRH input may be able to influence the stability and translatability of LHβ mRNA thus leading to the synthesis of the polypeptide chains of LH (Starzec *et al* 1986). Under physiological conditions, increasing oestradiol may lower the effective GnRH concentration required for this response (Ramey *et al* 1987; Huang and Miller 1980).

In the sheep oestrous cycle, a GnRH surge occurs concomitantly with the preovulatory LH surge (Moenter *et al* 1991, Clarke *et al* 1987). These investigators have found that the GnRH surge extended beyond the end of the LH surge. Moenter *et al* (1991) suggested that the elongated time scale of the GnRH surge may serve to down-regulate the GnRH receptor through continuous exposure (Nett *et al* 1981), and thus terminate the LH surge. A further role for this elevated level of GnRH may be to increase polyadenylation of the LHβ mRNA and thus increase the rate of replenishment of intracellular LH stores.

The rate of translation may also be affected by events which occur when the nascent peptide leaves the ribosome. The SRP, which binds to the newly translated protein product and allows the targeting of the entire complex to a specific receptor located on the RER membrane, is able to retard the extension of the same product from the

ribosome (Seigel and Walter 1988). If present in the gonadotroph, this effect may contribute to the initial lack of synthesised protein observed.

It is noteworthy that pulsatile LH release does not occur until 48h after the buserelin-induced LH surge. There are several potential explanations as to the absence of pulsatile LH release at B+24h. The paucity of LHβ immunopositive cytoplasmic granules and their appearance 48h after surge induction correlates with the establishment of pulsatile LH secretion. It is possible that LH, present in a non-granular form, is able to exit the cell in a constitutive fashion without an external exocytotic stimulus. In the present study, the measurement of a basal LH concentration of 1.03±0.11 ng/ml 24h after the induction of an LH surge, indicated that LH was still being released into the circulation and supports this hypothesis. The probable disrupted nature of the cortical cytoskeleton, following the LH surge, might assist in this releasing process. The fact that LH may not be contained within a granule structure may prevent the gonadotroph from co-ordinating intracellular hormone movements and therefore responding to any exocytotic stimulus. The synthesis of granules, visible at B+48h, may allow the synchronous movement of intracellular hormone stores and lead to a bolus exocytosis - the LH pulse. It has not yet been determined if GnRH pulses are present 24h after the LH surge. In this study, the lack of pulsatile LH release at B+24h may also be due to the absence of GnRH pulses.

Although the agranular form of LH is apparently only released in a constitutive fashion, it would appear that a co-ordinated bolus release is possible if a suitable stimulus is applied. LH secretion has been demonstrated using a GnRH agonist from 6h after the preovulatory LH surge in the ewe (Dobson *et al* 1974). Previous studies in ewes have also shown that treatment with GnRH agonist 24h after an induced LH surge produced a small bolus release of LH (Crighton and Foster 1976, 1977). The bolus release of LH was of similar magnitude to that released following GnRH agonist stimulation 12h after an induced LH surge (Crighton and Foster 1976, 1977). This suggests that whilst granule structure may be required in the physiological situation for the release of LH pulses, supraphysiological stimuli may be able to release agranular LH. Due to the low levels of agranular LHβ immunoreactivity observed at B+24h in the present study, it may be that the termination of the second induced small LH surge results from an emptying of cellular LH stores.

Changes in the sensitivity of the gonadotroph to the exocytotic stimulus will also influence secretory capacity. GnRH-binding activity rose from B+24h to B+72h thus

indicating the increased ability of the gonadotroph to respond to the LH-releasing ligand. Pulsatile LH release may involve a threshold phenomenon for which a certain level of stimulus, and therefore a certain number of GnRH receptors, must be present to induce exocytosis of sufficient LH to constitute a pulse. This could involve the directional nature of the calcium fluxes generated by the activation of the GnRH receptor (Anderson *et al* 1992). GnRH receptor down-regulation 16h after the LH surge has been reported (Brooks *et al* 1993).

Although the gonadotroph is less able to recognise the GnRH signal at B+24h, it may be that signal transduction is adequate but insufficient GnRH is present. However, it is likely that GnRH was released at this time of the cycle. GnRH is apparently required for the synthesis of LH. This requirement was first suggested many years ago when studies on male and female rats showed greatly decreased LH pituitary content in response to active immunisation against GnRH (Fraser *et al* 1974; Fraser *et al* 1975). Similar decreases in LH pituitary content were subsequently demonstrated on GnRH immunised ovariectomized ewes (Framer *et al* 1981) and ewes treated with GnRH agonist (McNeilly *et al* 1991). It is possible that the pituitary LH content measured at B+24h is not due to *de novo* synthesis of the hormone but residual stores not exocytosed during the preovulatory LH surge. This explanation is considered unlikely for two reasons, namely:

1. non-granular LH immunostaining was only observed at B+24h, being absent at all other stages of the cycle.
2. granular LH was virtually absent at B+24h, suggesting its contribution to the surge.

Increased LH pulse frequency and mean pulse amplitude from B+24h to B+96h indicate the rising secretory capacity of the gonadotroph cells throughout the refill phase. One or all of the previously suggested mechanisms may function in regulating this responsiveness.

The inability to synthesise secretory granules despite the LHβ immunoreactivity present may reflect the lack of a cellular product, the synthesis of which is decreased in the approach to the LH surge. The role of the class of acidic proteins known as the granins, which are colocalised within LH granules, will be investigated in Chapter 7.

CHAPTER 6

THE INFLUENCE OF GnRH AND OESTRADIOL ON GONADOTROPHIN GRANULE POLARISATION IN GONADOTROPHS DURING THE FOLLICULAR PHASE

Introduction

The studies outlined in this thesis to date have demonstrated that the ovine gonadotrophs polarise their LHβ immunopositive granule stores in the direction of the adjacent sinusoid, in preparation for the production of the preovulatory LH surge or pulsatile release of LH. The presence of polarised cells in varying quantities throughout the oestrous cycle, and the progressive recruitment of non-polarised gonadotrophs into the polarised cohort suggest that polarisation is required for co-ordinated release of LH.

The polarisation of the secretory granule body was always directed towards the vascular system. It is therefore possible that a signal, delivered via the vascular system, controls the direction and the extent of polarisation. As the LH surge is generated as a result of increasing GnRH pulse frequency (Clarke *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994) and increased oestradiol production from the developing follicle (Hauger *et al* 1977; Pant *et al* 1977; Baird 1978; Baird and McNeilly 1981), these two hormones are potential regulators of the morphological changes observed. It has been stated that the oestradiol-induced increase in GnRH receptor number does not account fully for the alteration in pituitary responsiveness to GnRH, and that oestradiol may modify post receptor mechanisms that influence LH secretion (Clarke *et al* 1988). In order to investigate the controlling mechanisms of polarisation, anti-oestradiol antibodies and GnRH antagonist together with oestradiol implants were use to determine the individual roles of these hormones in the polarisation process.

6.1. Materials and Methods

6.1.1. Animals and Experimental Design

The oestrous cycles of 36 Welsh mountain ewes were synchronised by withdrawal of intravaginal progesterone sponges as previously described (**2.2.1.**). Animals were allocated randomly into 5 groups (n=6 per group). Luteolysis was induced by the

intramuscular injection of $PGF_{2\alpha}$ and one group killed 36h after PG to act as follicular controls. At the time of PG injection, the other groups were treated with GnRH antagonist (Nal-Glu.HOAc, 5mg subcutaneous - a gift from the Contraceptive Development Branch, Centre for Population Research, NICHD), oestradiol implant (giving 5pg/ml of oestradiol), GnRH antagonist (5mg subcutaneous) plus oestradiol implant and anti-oestradiol antibodies (5ml intravenous), respectively. Chronically catheterised animals (**2.2.2.**) were sampled, initially at 8-hourly intervals from cannulation. In addition, frequent blood samples were taken at 10 minute intervals for the five hours immediately prior to death, to determine the pulsatile nature of the LH secretion from each animal.

6.1.2. Radioimmunoassay

Plasma LH concentrations and pituitary content were measured using the radioimmunoassay. The sensitivity of the plasma LH radioimmunoassay was 0.3ng/ml and the intra- and inter-assay coefficients of variation were 11.2% and 4.8% respectively. The sensitivity of the pituitary LH content assay was 2ng/ml and the intra-assay coefficient of variation was 11.6%. Plasma oestradiol concentrations were also determined by radioimmunoassay (assay sensitivity = 0.2pg/ml, intra-assay coefficient of variation = 12.9%). Immunoneutralisation of oestradiol was confirmed by binding of tracer. Assay details have been previously described (**2.2.3.**)

6.1.3. GnRH Binding

GnRH-binding activity was determined using pituitary homogenates as previously described (**2.2.4.**)

6.1.4. Measurement of mRNA Abundance

Following the removal of a slice of tissue for immunocytochemistry, the remainder of the pituitary was frozen in liquid nitrogen. Total RNA was extracted and subjected to Northern analysis using cDNA probes for LHβ (**2.2.7.**). The amount of RNA present was quantified using a Phosphor Imager (Molecular Dynamics).

6.1.5. Ultrastructural Immunocytochemistry

Animals were humanely killed and pituitaries removed immediately. Following removal of excess connective tissue, whilst the pituitary remained on ice, a 1mm thick slice from the mid-region of the adenohypophysis was immersion-fixed in 4% paraformaldehyde/0.1% glutaraldehyde and then processed and sectioned for immunocytochemistry under the transmission electron microscope as previously described (**2.2.6.2.**). Gonadotrophs were identified by the immunogold labelling of LHβ (**2.2.6.3.**(b)). Percentages of polarised gonadotrophs were determined by counting twenty cells per animal, located by systematic random sampling.

6.2. Results

6.2.1. Plasma and Pituitary LH Levels/ GnRH Binding

Radioimmunoassay of blood samples showed that GnRH antagonist and GnRH antagonist plus oestradiol treatments significantly ($P<0.05$) suppressed plasma LH levels (Fig 67). In other treatment groups, LH concentrations did not differ significantly from control values.

Parameters of pulsatile LH secretion are shown in Table 6.

Table 6. LH pulse frequency, pulse amplitude and basal concentrations in follicular phase control ewes and ewes treated with GnRH antagonist, GnRH antagonist plus oestradiol, oestradiol and anti oestradiol at the time of prostaglandin induced luteolysis, 36h before death. Values are given as mean ± SEM and n = 6 in all groups. Pulse frequencies are expressed as pulses/6h to allow direct comparison between these and pulse frequencies in Chapters 4 and 5. * indicates a significant ($P<0.05$) decrease in pulse frequency from follicular controls.

Treatment Group	Pulse Frequency (Peaks/6h)	Pulse Amplitude (ng/ml)	Basal LH (ng/ml)
Follicular Control	5.4 ± 0.96	1.02 ± 0.23	0.92 ± 0.12
GnRH Antagonist	0	0	0.59 ± 0.03
GnRH Antagonist plus Oestradiol Implant	0	0	0.51 ± 0.06
Oestradiol Implant	2.4 ± 1.08*	1.07 ± 0.52	1.02 ± 0.26
Anti-Oestradiol	3.24 ± 1.32	1.44 ± 0.37	0.93 ± 0.01

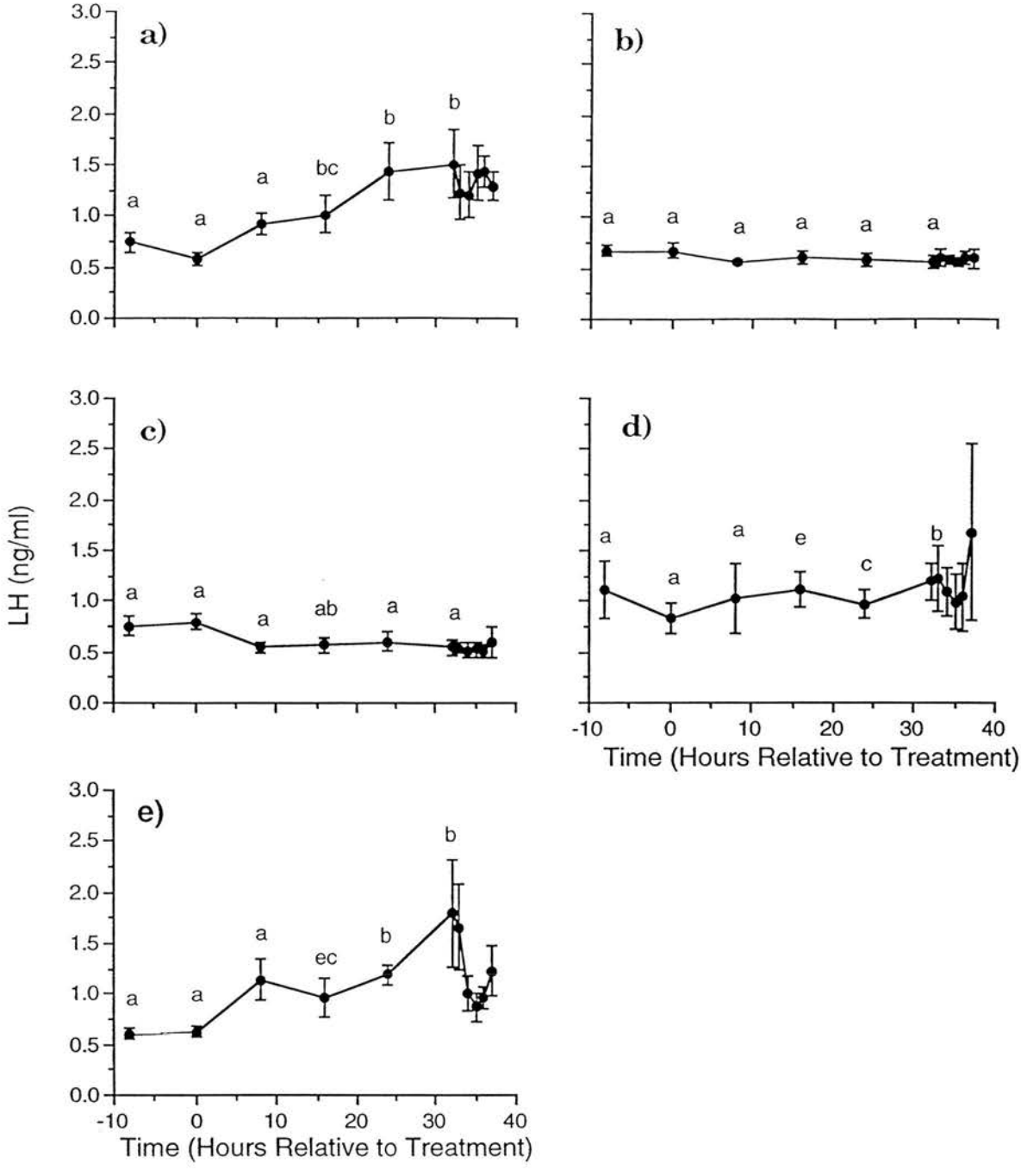


Figure 67. Changes in plasma concentrations of LH during the follicular phase after prostaglandin-induced luteolysis (0h) in treatment groups a) untreated controls, b) GnRH antagonist (5mg subcutaneous) c) GnRH antagonist plus oestradiol implant (3mm subcutaneous) d) oestradiol implant alone or e) sheep anti-oestradiol serum (10ml intravenous). Values are mean ± SEM, n=6 per group. Different letters indicate significant ($P<0.05$) differences between treatment groups at each sampling point.

The GnRH antagonist and GnRH antagonist plus oestradiol abolished LH pulses completely. Oestradiol treatment significantly ($P<0.05$) decreased LH pulse frequency over control values whilst anti-oestradiol caused no significant alteration in pulse frequency. When LH pulses were present, the amplitudes did not differ significantly from control values. The two groups receiving GnRH antagonist possessed basal values significantly lower than those present in the control, oestradiol and anti-oestradiol groups. Representative LH pulse profiles from individual ewes from each treatment group are shown in Figure 68.

The pituitary LH content measurement for GnRH antagonist, oestradiol treatment and GnRH antagonist plus oestradiol did not differ significantly from control values (Fig 69a). A slight suppression of LH content was observed after anti oestradiol treatment but this did not reach a level of significance.

Treatment with GnRH antagonist and GnRH antagonist plus oestradiol caused a significant ($P<0.05$) reduction of GnRH-binding (Fig. 69b) activity over follicular control levels. The oestradiol implant and anti-oestradiol also caused a significant ($P<0.05$) decrease in GnRH-binding over controls but this level was still significantly ($P<0.05$) greater than the GnRH antagonist treatment groups.

Plasma oestradiol concentrations showed a significant ($P<0.05$) increase between control and oestradiol implant groups, whilst GnRH antagonist caused a significant ($P<0.05$) decrease from control values (Fig. 70a). The significant ($P<0.05$) increase in the binding of tracer to plasma samples demonstrated the presence of increased amounts of anti-oestradiol antibodies following their intravenous administration (Fig.70b).

6.2.2. Measurement of mRNA Abundance

Treatment with GnRH antagonist, oestradiol implant and GnRH antagonist plus oestradiol implant significantly ($P<0.05$) decreased LHβ mRNA from follicular control values (Fig. 71). The levels measured in these treatment groups did not differ significantly from each other. Anti-oestradiol caused a non significant suppression of steady state LHβ mRNA from follicular control values.

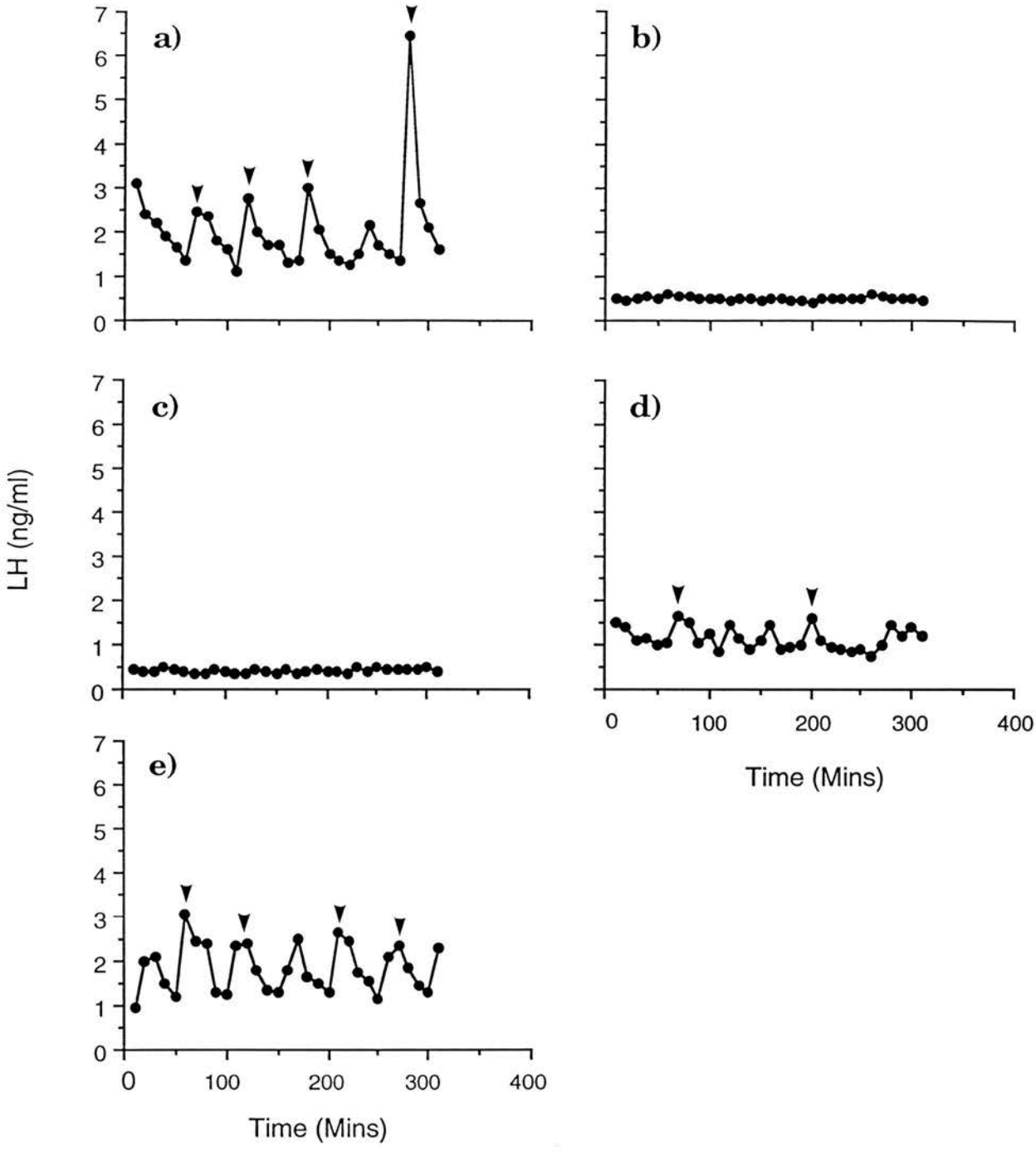


Figure 68. Representative profiles of pulsatile LH secretion from individual ewes during the follicular phase after prostaglandin-induced luteolysis (0h) from treatment groups a) untreated controls, b) GnRH antagonist (5mg subcutaneous) c) GnRH antagonist plus oestradiol implant (3mm subcutaneous) d) oestradiol implant alone or e) sheep anti-oestradiol serum (10ml intravenous). Arrowheads indicate significant LH pulses.

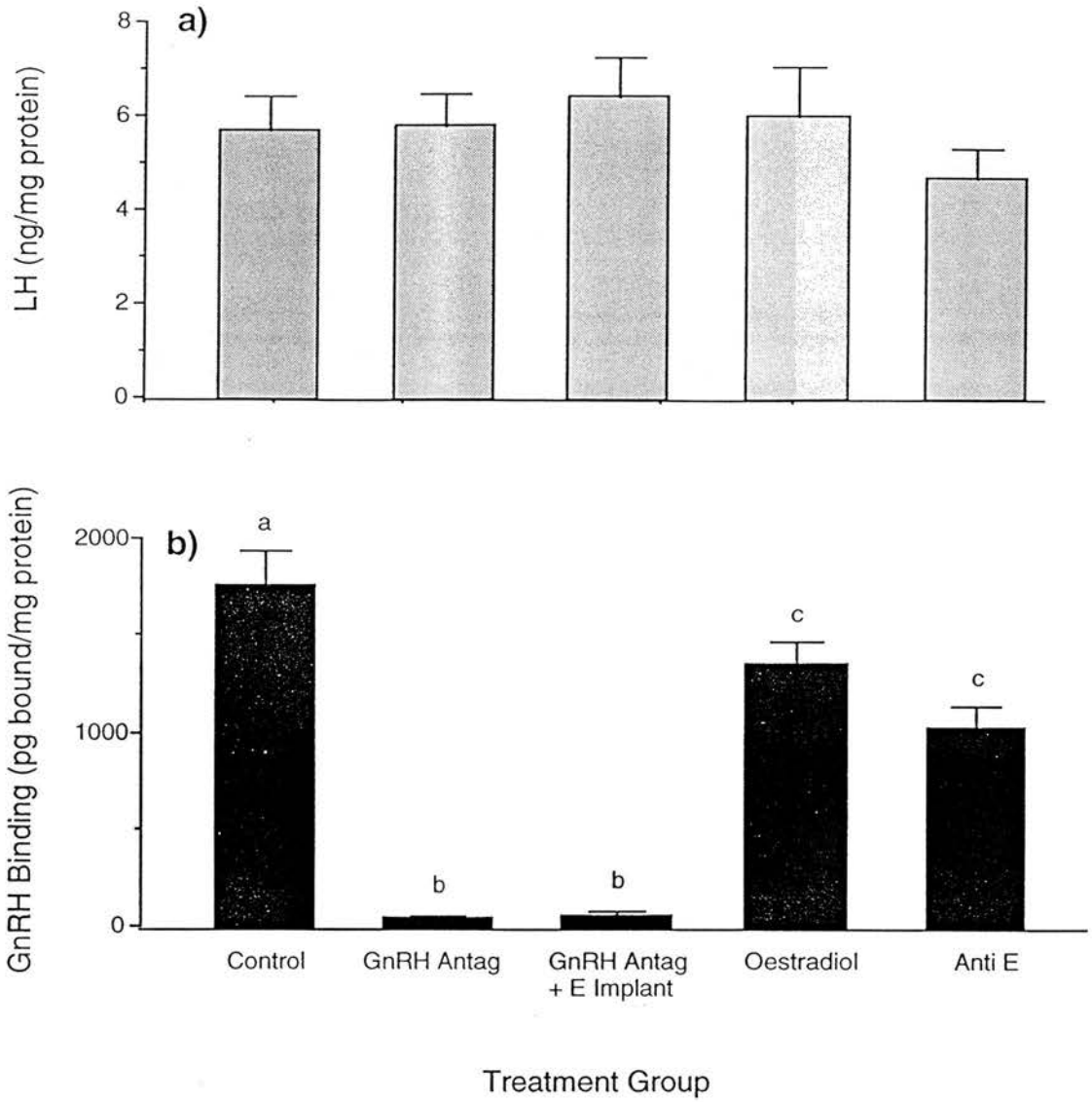


Figure 69. Changes in a) pituitary LH content and b) GnRH-binding activity in follicular phase control ewes and ewes treated with GnRH antagonist, GnRH antagonist plus oestradiol, oestradiol and anti-oestradiol at the time of prostaglandin-induced luteolysis, 36h before death. Data was analysed by one-way ANOVA. Values are given as mean ± SEM and different letters denote significant ($P<0.05$) differences.

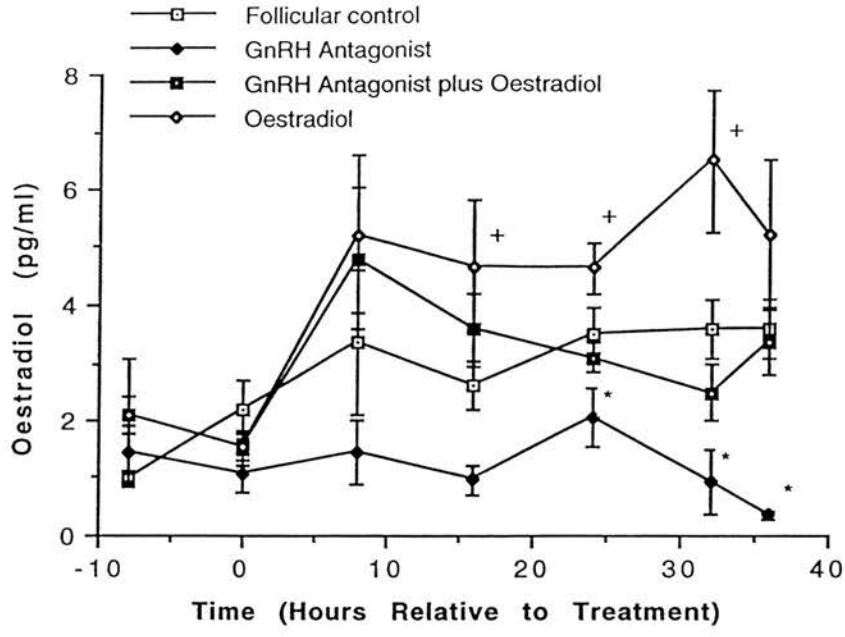
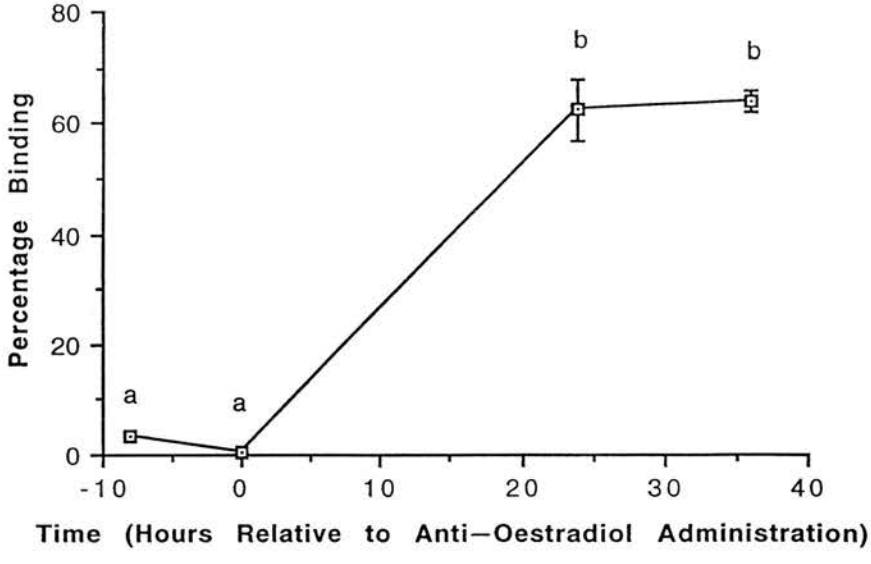


Figure 70. a) Changes in the plasma concentrations of oestradiol in follicular control, GnRH antagonist, GnRH antagonist plus oestradiol and oestradiol treatment groups. Concentrations are given as mean ± SEM and data analysed by one-way ANOVA. + = significant ($P<0.05$) difference between control and oestradiol treatment groups. * = significant ($P<0.05$) difference between control and GnRH antagonist treatment groups.



b) Oestradiol binding capacity (at a dilution of 1:400) in the plasma of ewes injected at the time of prostaglandin-induced luteolysis (0h) with a 10ml intravenous bolus injection of sheep anti-oestradiol serum. Results are mean ± SEM and data was analysed by one-way ANOVA. Different letters denote a significant ($P<0.05$) difference.

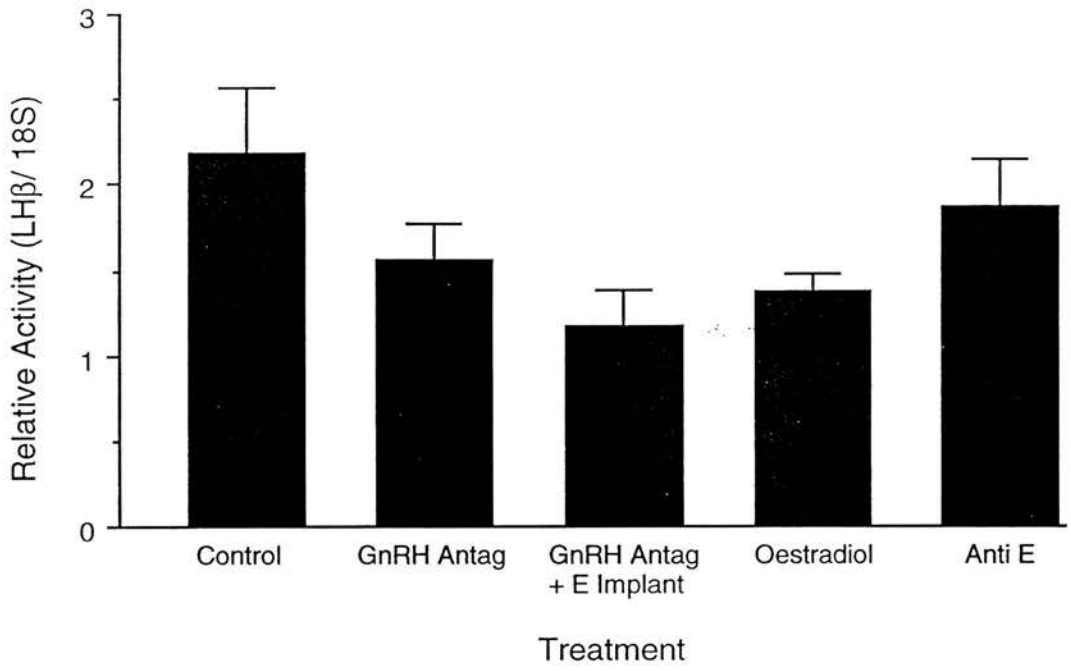


Figure 71. Changes in LHβ mRNA abundance from follicular phase control ewes and ewes treated with GnRH antagonist, GnRH antagonist plus oestradiol, oestradiol and anti oestradiol at the time of prostaglandin induced luteolysis, 36h before death. Results are expressed relative to 18S ribosomal mRNA subunit abundance. Blots were exposed for 2h and the results quantified using a Phosphor Imager. Values are given as mean ± SEM, n=6 per group. Differences in LHβ mRNA abundance were not significant (one-way ANOVA).

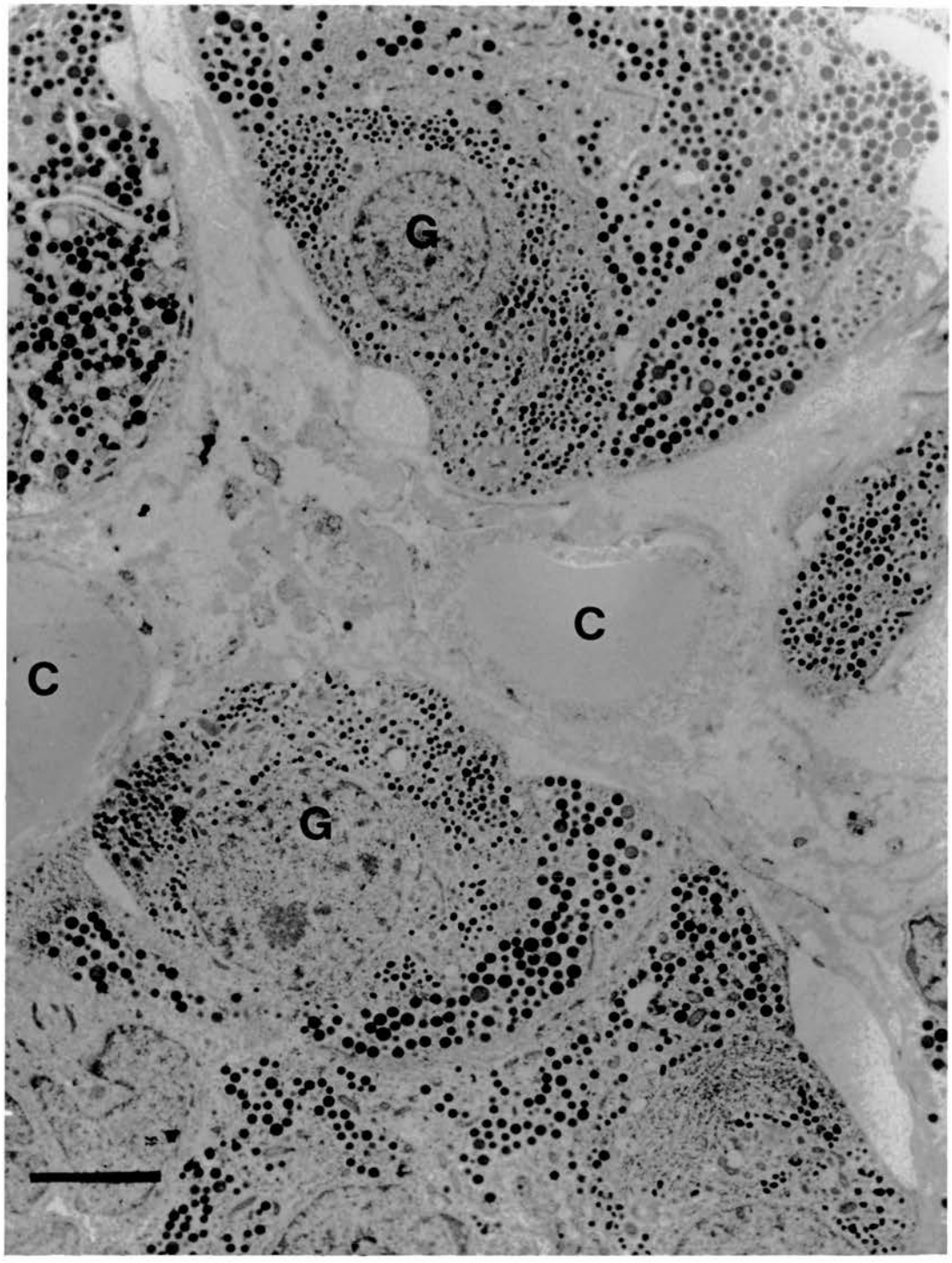
6.2.3. Ultrastructural Observations

Immunogold localisation of LHβ subunit identified gonadotrophs in all the animals in the study. Follicular phase control animals displayed a mean of 80±3.65% non polarised gonadotrophs (Fig. 72) with the remaining 20±3.65% exhibiting a classically polarised morphology, as defined by the orientation of the granule body towards the adjacent sinusoid (Fig. 73). Treatment of animals with GnRH antagonist resulted in 75±3.4% non-polarised gonadotrophs with 25±3.4% in the polarised state. These differences were not significant. Of this 25%, 7.5±4.1% were polarised irregularly in that the granule body did not appear directed exactly towards the juxtaposed sinusoid (Fig. 74). Oestradiol administration significantly ($P<0.05$) reduced the non-polarised subpopulation to 41.65±2.4% with the remaining 58.35±2.4% of the gonadotrophs polarised. The polarised cohort contained 20.0±3.4% irregular polarised cells, significantly ($P<0.05$) greater than values observed with GnRH antagonist. GnRH antagonist plus oestradiol resulted in 41.65±2.4% polarised gonadotrophs of which 16.65±3.35% were polarised irregularly (Fig. 75), whilst 58.35±4.6% were non-polarised. Finally, the administration of anti-oestradiol produced 54.15±2.7% polarised gonadotrophs containing 15.85±2.4% irregular polarised cells. (Fig. 76). The non-polarised population in these animals comprised 45.85±2.7%. The percentages of irregular polarised cells in the oestradiol, GnRH antagonist plus oestradiol and the anti-oestradiol antibody treatment groups did not differ significantly and there were no obvious relationship between treatment and any particular irregularly polarised granule distribution.

6.3. Discussion

The down-regulation of LHβ mRNA observed following treatment with GnRH antagonist demonstrates that GnRH is required for the transcription of LHβ mRNA. This is in agreement with previous studies in rats which have shown that *in vitro* GnRH stimulates the synthesis of the polypeptide chains of LH subunits (Starzec *et al* 1986) and increases levels of LHβ mRNA (Weirman *et al* 1989). From the studies carried out during luteal through the follicular phase this is not the predominant mechanism operating during the follicular phase of the ovine oestrous cycle, since a down-regulation of LHβ mRNA was observed despite the increasing GnRH pulse frequency present as the LH surge approaches (Moenter *et al* 1991). The decreased levels of LHβ mRNA due to oestradiol treatment both in the presence and absence of GnRH suggest that oestradiol may control the decline in mRNA levels throughout the follicular phase

Figure 72. This transmission electron micrograph shows immunoidentified gonadotrophs (G) in an animal from the follicular control group. The most gonadotrophs displayed non-polarised granule distributions with respect to the adjacent capillaries (C). Magnification = 8100X. Scale bar = 2.5μm.


G
C
C
G

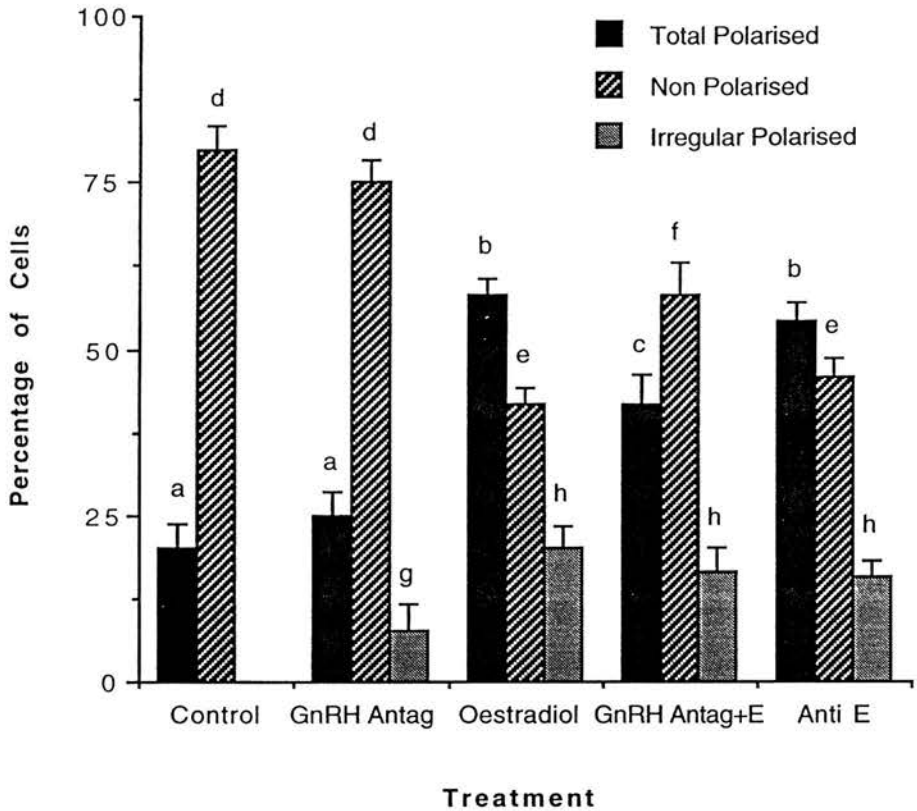


Figure 73. Changes in percentages of immunoidentified gonadotroph cells with polarised granule distributions in follicular phase untreated control ewes and follicular phase ewes treated with GnRH antagonist, GnRH antagonist plus oestradiol, oestradiol and anti-oestradiol. Percentages are given as mean ± SEM and data analysed by one-way ANOVA. Different letters denote a significant ($P<0.05$) difference.

Figure 74. Treatment with GnRH antagonist led to the appearance of polarised cells with granule distributions slightly different to that observed during the oestrous cycle. This transmission electron micrograph shows an immunoidentified gonadotroph (G) with adjacent sinusoid (S) and endothelial cell nucleus (EN). Although some gonadotrophin secretory granules were polarised in the direction of the vascular system, others (↟) remained in the aspect of the cytoplasm furthest from the vascular system. Magnification = 8900X. Scale bar = 2.2μm.

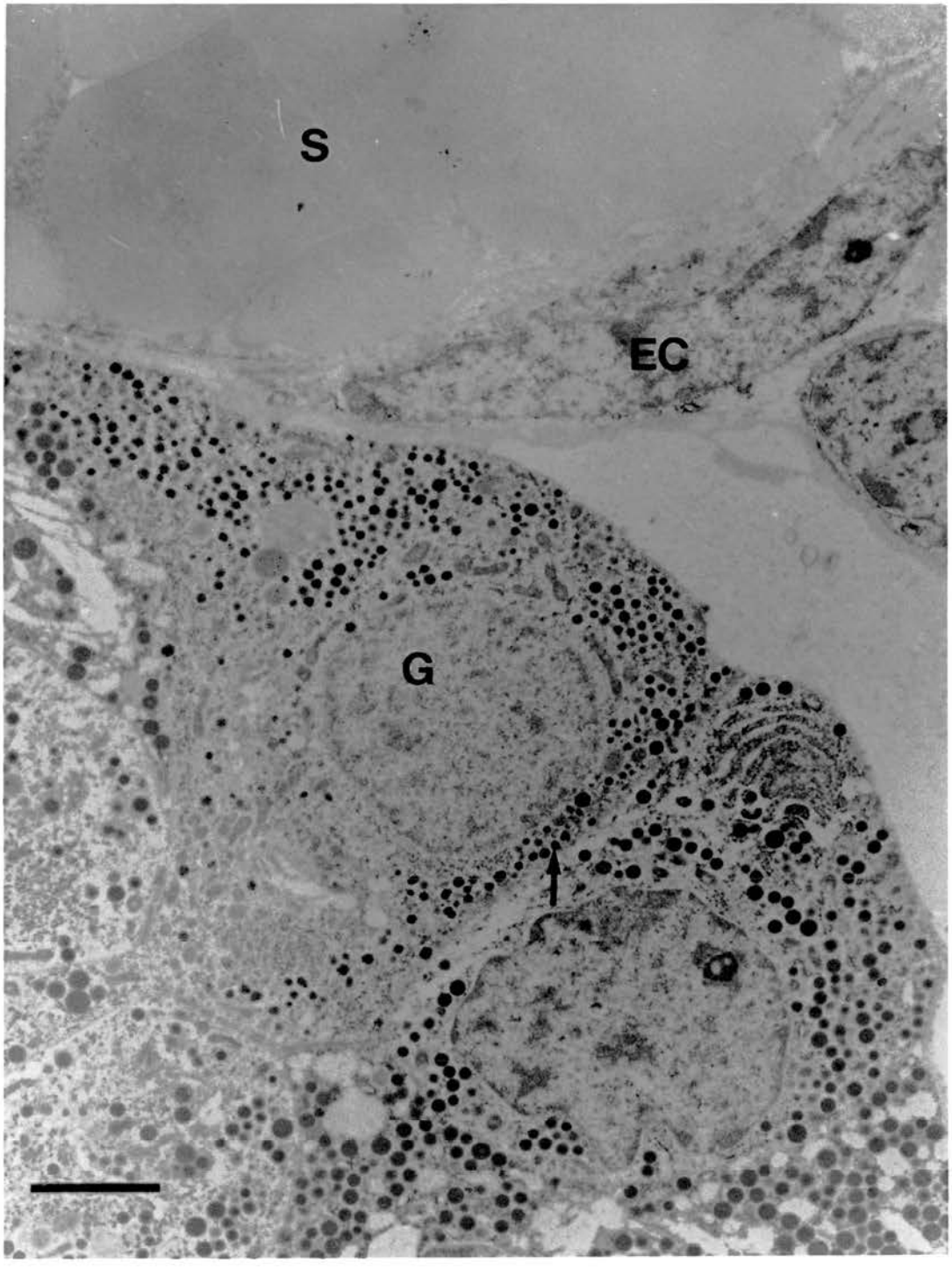

S
EC
G

Figure 75. Transmission electron micrograph showing an immunoidentified gonadotroph (G) in an animal treated with GnRH antagonist and oestradiol. In this cell, the polarisation of the granule body was directed in the opposite direction to the vascular system, as defined by the position of the endothelial cell nucleus (EN). Magnification = 11500X. Scale bar = 1.7μm.

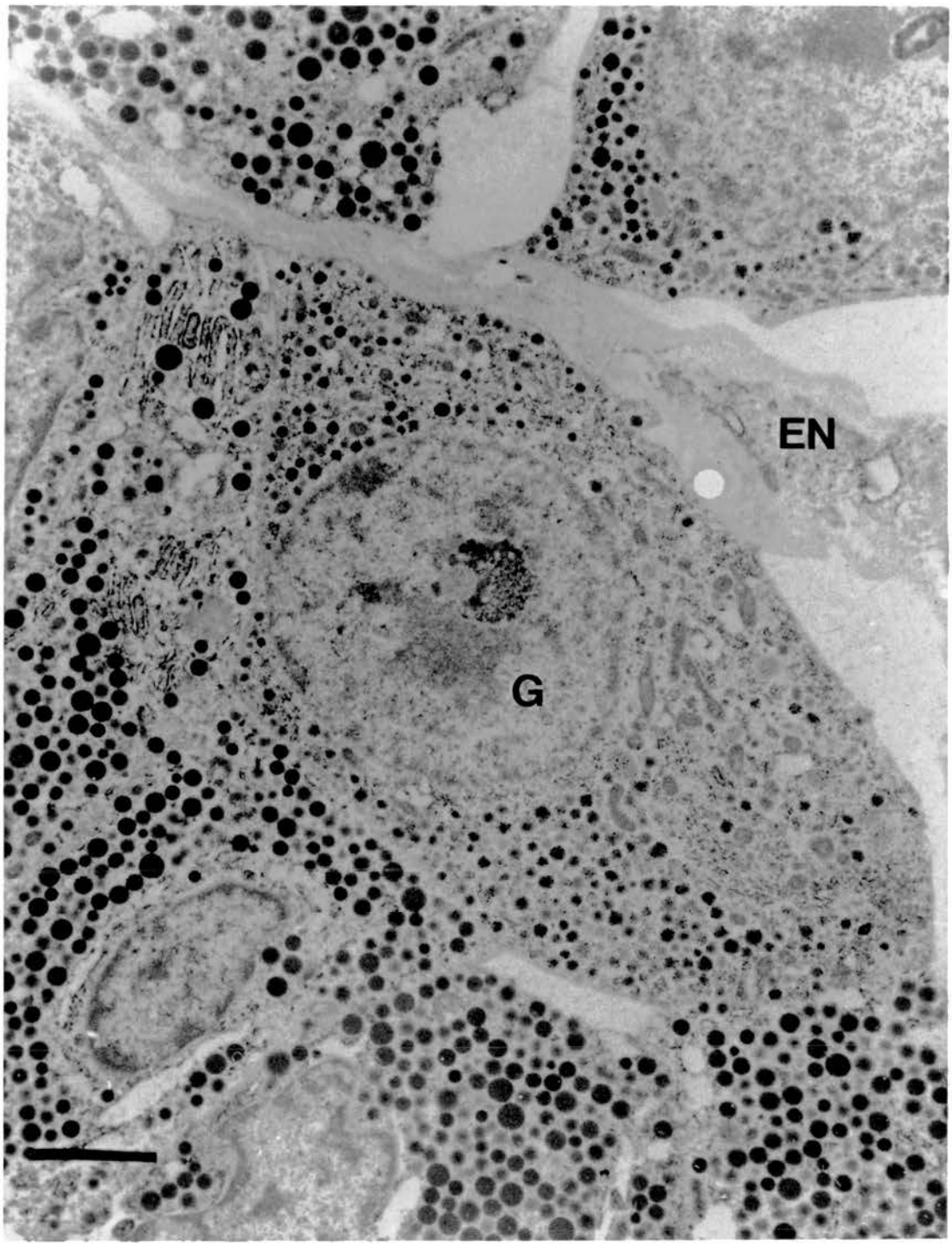
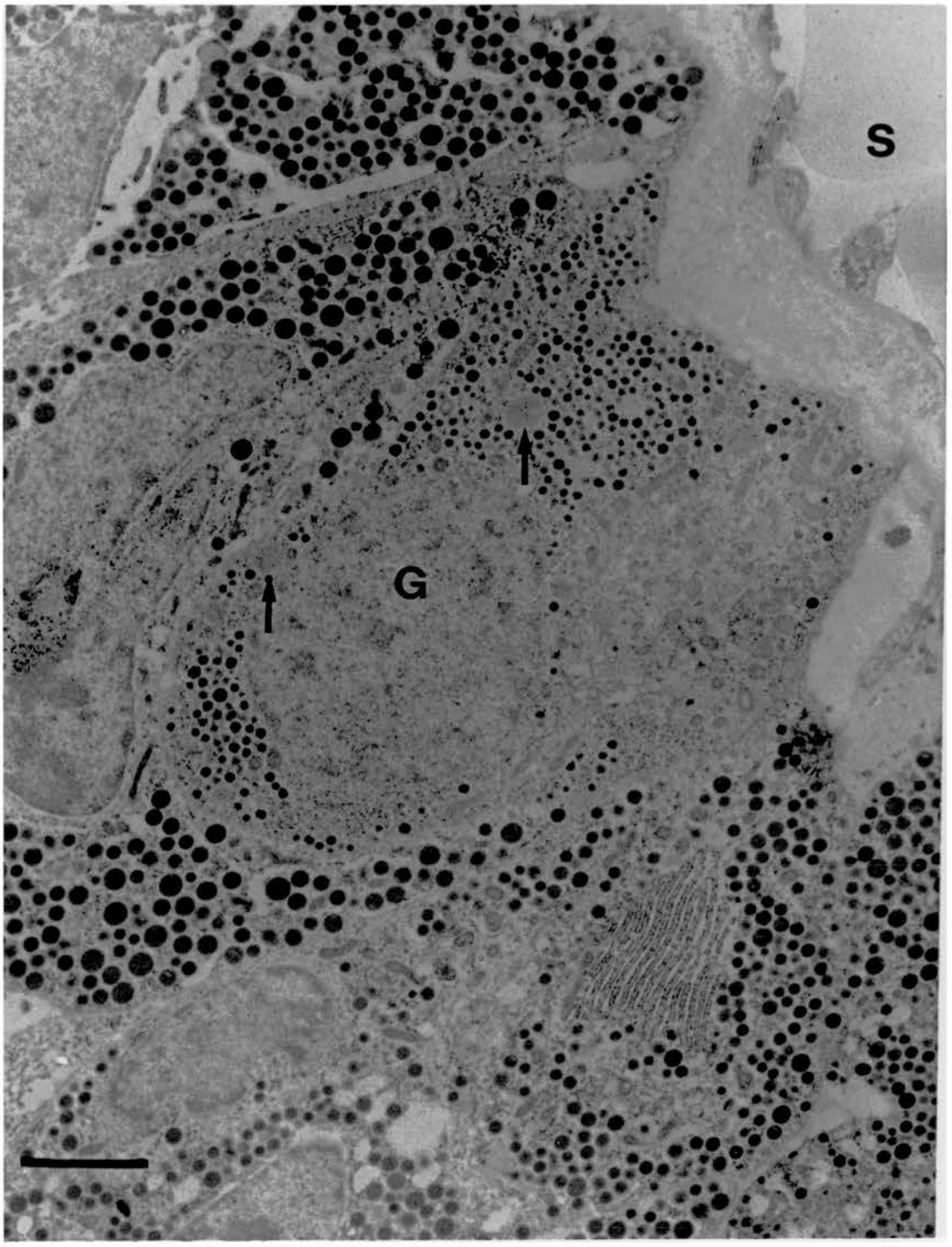

EN
G

Figure 76. Irregular polarisations were also observed in gonadotrophs from animals treated with anti-oestradiol antibodies. In this transmission electron micrograph of an immunoidentified gonadotroph (G), secretory granules were polarised to one aspect of the cell at 90^{o} to the sinusoid (S). Light dense bodies (↟) were only localised with the granule body. Magnification = 8100X. Scale bar = 2.5μm.


S
G

(Chapter 4). This negative action of oestradiol has been shown previously in sheep pituitary: using tissue from ovariectomized ewes, levels of mRNA and translated β subunit were elevated two to three times over control values using cell-free translation assays (Landefeld and Kepa 1984a); oestradiol suppresses accumulation of LHβ and common α subunit mRNAs (Nilson *et al* 1983, Jutisz *et al* 1988).

In contrast to these findings, observations in the rat suggest that LHβ mRNA abundance increases throughout the oestrous cycle, reaching peak levels at the preovulatory LH surge during pro-oestrus (Zmeili *et al* 1986; Kakar *et al* 1993b). Furthermore, LHβ mRNA abundance has also been shown to increase during the preovulatory LH surge in the sheep (Landefeld 1985; Leung *et al* 1988). Although Landefeld *et al* (1989) suggested this rise may be due to oestradiol, they were unable to elicit a significant increase in LHβ mRNA when oestradiol was administered to anoestrus ewes. Similarly, Brooks *et al* (1993) reported no change in LHβ mRNA abundance throughout the ovine oestrous cycle. An oestradiol-induced LH surge in the rat has been reported without any change LHβ mRNA (Haisenleder *et al* 1988).

The anomaly between the results of this study and those in the rat is perhaps a consequence of the different lengths of oestrous cycles present in the two species. Co-ordinated movement of the entire granule body may require LH synthesis to be completed before polarisation progresses. Furthermore, the present study suggests that the positive effects of oestradiol on LH secretion in the sheep may be achieved by positive feedback on the GnRH pulse generator (Karsch *et al* 1980b; Goodman and Karsch 1980), together with the movement of intracellular stores to a cellular location which favours release.

The differences between the decrease in LHβ mRNA throughout the follicular phase of the ovine oestrous cycle in this study, and the observations made by Landefeld (1985) are difficult to explain. Certainly, mRNA abundance was determined in different ways: the present study used the hybridisation of radioactively labelled cDNA probes and quantified the signal obtained on a Northern blot with the use of a Phosphor Imager; the other investigators also used radioactively-labelled cDNA probes but determined RNA abundance by the comparison of slopes derived from linear regression plots of radioactive counts per minute versus three different concentrations of RNA. Only single representative animals were assessed. From the graph of linear regressions published by Landefeld (1985), it is notable that the intercept of regression lines does not occur at the same point on the y axis. The authors suggest that this is due to 'minor differences in the amount of background and other variables in the blot assay such as transfer

efficiency'. Differences in the dilution of RNA for blotting analysis would also account for these differences. It is highly significant that the greatest difference in zero intercept values occurred between the reference value (day 12) and E+25, which were also the time points where the greatest differences in mRNA abundances were observed. The absolute difference in the intercept values was some $1.2x10^{-3}$ counts per minute, around 20% of the maximum counts calculated for E+25. The movement of the E+25 intersect by this amount would greatly decrease the gradient, therefore removing most of the observed difference in RNA abundance. The analysis of greater numbers of animals would have given a measure of dispersion which is also absent from the data. Although it seems unlikely to be the cause of major differences in values, the present study was conducted on Welsh mountain ewes whereas Landefeld and co workers used Suffolk ewes. Finally, although both methods attempt to assess mRNA abundance, neither measures the actual quantities of RNA undergoing translation. Thus it is possible that differing amounts of RNA could result in the same degree of protein synthesis. This will require further investigation.

The treatment of ewes with oestradiol, GnRH antagonist plus oestradiol, and anti-oestradiol increased the numbers of polarised cells present over follicular control values. These three treatments together with the GnRH antagonist also induced an irregular polarisation effect in that the granule body appeared mis-directed in a number of instances. Although granules were located specifically in one part of the cytoplasm, they were not always juxtaposed to the nearest capillary. LHβ steady state mRNA abundance was suppressed in all treatment groups compared to follicular control levels as was GnRH binding activity. The suppression of GnRH binding activity following GnRH antagonist treatment, in isolation and in combination with oestradiol, was significantly ($P<0.05$) greater than that attained by oestradiol and anti-oestradiol treatments respectively. GnRH-binding was reduced in all groups when compared to the follicular control levels whilst GnRH antagonist treatment abolished LH pulsatility and decreased the mean LH basal level. GnRH antagonists have previously been shown to completely inhibit pulsatile LH secretion within 1h of injection (Campbell *et al* 1990).

The GnRH binding data confirmed the efficacy of the antagonist, the decreased level of binding attributable to the antagonist occupying the GnRH-binding sites in the preparation and thus preventing the ligand from interacting. The binding of the antagonist to the gonadotroph cell membrane prevents the exocytotic stimulus, in the form of GnRH, from acting through its receptor, leading to the abolishment of LH pulses observed. The down-regulation of LHβ mRNA observed did not result in any measured decreases in pituitary LH content. This is consistent with the hypothesis that the

gonadotroph remains in the main synthetic state during luteal phase. As the LH surge approaches, the synthesis decreases and the main events centre around the post-translational control of previously synthesised LH stores. The down-regulation of the LHβ mRNA through the follicular phase thus duplicates the expected physiology occurring at that time, leading to no changes in pituitary content.

The follicular phase control group gives an indication as to the time course of the polarisation event. At 36h after luteolysis, polarised cell percentages were similar to those observed in luteal and early follicular phase controls but less than at behavioural oestrus (**4.2.**). The observation was made despite the fact that LH pulse frequency had increased to levels previously observed at oestrus+9h (**4.2.**) and that plasma oestradiol levels were equivalent to the GnRH antagonist plus oestradiol group (where polarised cell percentages were greater). It is possible that the failure to increase the polarised cell cohort, despite the increased GnRH input, was due to there being insufficient time for the hormone to act before the animals were killed. Behavioural oestrus where approximately 40% of gonadotrophs were polarised was shown to occur at 33.6±0.98h after luteolysis (**4.2.**). However, other studies on the Welsh mountain ewe have shown behavioural oestrus to occur 38.8±2.3h after PG (Picton 1989). Thus, it is possible that animals may not have entered behavioural oestrus 36h after PG which may explain the lower percentage of polarised cells reported in this chapter. The time between behavioural oestrus and the onset of the LH surge of approximately 9h (**4.2.**) may be a reflection of the time of exposure to GnRH required to induce the extensive granule polarisation that accompanies the LH surge. The additional percentage of polarised cells present in the GnRH antagonist plus oestradiol group, despite the absence of GnRH pulses and a similar oestradiol level to the follicular control group, may be due to two factors:

1. the initial bolus release of oestradiol from the implant, as indicated by plasma oestradiol levels, may increase the rate of recruitment of polarised gonadotrophs.
2. the action of the GnRH antagonist on the cellular receptor may elicit a non-specific membrane phenomenon, leading to premature polarisation.

The possibility of a non-specific membrane phenomenon is suggested by the fact that the GnRH antagonist treatment group, despite a lack of LH pulses due to greatly reduced GnRH-binding, contained a slightly increased percentage of polarised cells. Also, this group contained a proportion of irregular polarised cells which were absent from the follicular controls.

In animals treated with anti-oestradiol antibodies, the gonadotrophs were only exposed to GnRH input, the oestradiol, produced as a result of the action of released LH on the ovary, being immunoneutralized. GnRH antagonist removes the exocytotic stimulus to

LH release. Therefore, oestradiol levels declined as a result of the lack of LH drive to the ovary. To ascertain the effect of oestradiol alone, it was necessary to replace the missing oestradiol using an implant. Individually, oestradiol and GnRH were able to elicit polarisation of the granule stores. Assuming that the polarised form of the gonadotroph is the releasing form (Chapter 4), this observation is in keeping with previous studies which have demonstrated that oestradiol is a modulator of the increased LH pulse frequency present in the follicular phase of the ovine oestrous cycle (Karsch *et al* 1983). If the hypotheses that granules are required for co-ordinated LH release and that polarisation is required before pulsatile LH release can be achieved are correct, then the surge-like release produced by these two hormones would require polarisation of the granule stores. The fact that mis-direction of the LH-immunopositive granules is evident in around 30% of cases, and the previous observation that during the oestrous cycle granules always appear polarised towards the vascular system, suggest that *in vivo* oestradiol and GnRH synergise to ensure the correct orientation of the polarisation.

There are several mechanisms that could control the polarisation process. Clearly, a vascular signal may be important in directing the granules towards the appropriate side of the cell. The means by which this signal is transduced to the granule body are still unclear. The cytoskeleton is a likely controller of the movement of intracellular organelles such as secretory granules (Adams and Nett 1979). If this is the case in the gonadotroph, a mechanism must be present which orientates the cytoskeleton. In the case of cytoskeletal microtubules, low calcium concentrations induce disassembly which is mediated by calmodulin (Marcum *et al* 1978, Keith *et al* 1986). Microtubule preparations, isolated from rat brain, contain a calcium/calmodulin dependent protein kinase. This enzyme is localised to tubulin, neurofilaments and microtubule-associated protein 2 (MAP-2), and is the likely effector of the calcium/calmodulin actions previously described. Interestingly, a calmodulin-binding protein has been identified on the chromaffin granule membrane thus providing a means by which a secretory granule could associate with areas of the cytoskeleton being remodelled (De-Block *et al* 1990). Control of cytoskeletal remodelling may be achieved by kinase-mediated phosphorylation of cytoskeletal proteins (Vallano *et al* 1986). Specifically, the calcium stimulated phosphorylation of MAP-2 decreases microtubule assembly, and may promote disassembly (Yamauchi and Fujisawa 1983). In contrast, phosphorylation of neurofilaments appears to stabilise their structure (Sternberger and Sternberger 1983). If the calcium-dependent kinase on the neurofilaments of neurones is similarly localised on the microfilaments of gonadotrophs, this enzyme may play a critical role in the polarisation process. It has been shown that the GnRH priming effect can be duplicated

by retreating perifused pituitary cell cultures with phorbol ester prior to GnRH challenge (Turgeon and Waring 1986). As phorbol esters function by activating protein kinase C (PKC), this suggests that PKC may also function in any phosphorylation processes involved in polarisation.

Studies on the GnRH receptor in an immortalised α T3-1 pituitary gonadotroph cell line have demonstrated a biphasic rise in intracellular calcium upon receptor activation (Clapper and Conn 1985; Leong *et al* 1986; Chang *et al* 1986; Turgeon and Waring 1986; McArdle *et al* 1992; Anderson *et al* 1992). The biphasic response is composed of an initial calcium spike, generated predominantly from the mobilisation of calcium stores from the endoplasmic reticulum (ER), followed by a plateau phase maintained by the influx of extracellular calcium via protein kinase C-activated calcium channels located in the plasmalemma. A polarised distribution of calcium channels and ER calcium stores would facilitate the stabilisation of distinct areas of the cytoskeleton. It is possible that this calcium flux may act via a kinase enzyme, stabilising the microfilaments in a desired orientation thus providing a framework for granule transport. The association of the granule membrane with actin filaments has been demonstrated (Burridge and Phillips 1975). The fact that microtubules disassemble in elevated intracellular calcium conditions indicates that their role in granule transport may be less critical. In dispersed ovine pituitary cells, disruption of the microfilament network reduced GnRH-stimulated LH release, whilst microtubule perturbation had no effect (Adams and Nett 1979). Thus, GnRH may polarise the gonadotroph through cytoskeletal stabilisation in a certain direction.

Oestradiol-induced granule polarisation may be achieved via a similar mechanism. It has been shown that *in vitro* stimulation of breast cancer cells with oestrogen and tamoxifen leads to cytoskeletal remodelling (Sapino *et al* 1986). Although tamoxifen is an anti-oestrogen, the same cytoskeletal reorganisation occurred with both agents. This may be attributed to the known partial agonist effects of tamoxifen *in vivo* (Patterson 1981). Further *in vitro* studies have described the interaction of the oestradiol receptor with calcium and calmodulin (Castoria *et al* 1988). Thus oestradiol, bound to its cytosolic receptor may be able to activate calmodulin, under the influence of calcium, and hence the kinase-mediated phosphorylation of the microfilaments, leading to their stabilisation. Furthermore, the calcium-mediated association of the oestradiol receptor *in vitro* required calcium levels above that of basal values (Castoria *et al* 1988). Gradually increasing intracellular calcium concentrations throughout the follicular phase of the oestrous cycle, due to increased GnRH/LH pulse frequency, may produce a more efficient interaction of the oestradiol receptor complex with calmodulin. The

greater stability attained in the microfilament cytoskeleton may allow a more effective polarisation. Although the positive feedback effects of oestradiol on gonadotrophin secretion have been attributed to an up-regulation in GnRH receptor number (Gregg and Nett 1989), cytoskeletal stabilisation and hence granule polarisation may be a further mode of action. Oestradiol may also act by stimulating the synthesis of microfilaments as the positive effect of oestradiol on GnRH mediated LH release is abolished by transcription and translation inhibitors, whilst the negative effect was unaltered (Emons *et al* 1989). The rise in plasma LH following passive immunisation against oestradiol, observed by Mann *et al* (1989), is likely to be due to the removal of the inhibitory effects of oestradiol on LH secretion, as immunoneutralization took place 24h after luteolysis in early follicular phase. This is suggested by the fact that large numbers of non-polarised gonadotrophs were observed in the present study 36h after PG-induced luteolysis. It is postulated that the disassembly of microtubules, whilst not critical to achieve successful exocytosis, may remove a degree of steric hindrance to granule transport thus allowing more efficient granule translocation. During the oestrous cycle, the actions of GnRH and oestradiol are linked in that increasing levels of oestradiol lead to an increase in GnRH receptor mRNA abundance (Brooks *et al* 1993; Wu *et al* 1994) and a resultant increase in GnRH receptor number (Clarke *et al* 1988; Brooks *et al* 1993) during the periovulatory period in the ewe (Crowder and Nett 1984). Furthermore, oestradiol lowers the concentration of GnRH necessary to stimulate the biosynthesis and glycosylation of LH α and β subunits thus sensitising the gonadotroph to the effects of GnRH (Huang and Miller 1980; Ramey *et al* 1987). These mechanisms provide an explanation of the apparent synergistic effects of GnRH and oestradiol on granule polarisation observed *in vivo*.

It was notable that both oestradiol and anti-oestradiol treatment produced similar changes in pituitary LH content, GnRH-binding activity and LHβmRNA levels. Although the mean values following anti-oestradiol treatment were lower than those attained with oestradiol, these differences did not reach a level of significance. Of these two treatments, anti-oestradiol produced an apparent higher LH pulse amplitude, but this difference was not significant. Polarised cell numbers were extremely similar following both treatments. These observations, together with the high oestradiol binding capacity of the plasma after anti-oestradiol administration, suggest that oestradiol complexed with antibody may still possess a slight affinity for its cytosolic receptor and hence retain some oestrogenic activity. Furthermore, the antibody-oestradiol complex may be able to elicit non-specific effects via cell membrane perturbation, influencing both the granule distribution within the cytoplasm and the synthetic status of the cell.

In the rat, exocytosis has been visualised almost exclusively at the vascular border of the gonadotroph (Durnin and Morris 1992a). The association of the granule body with the aspect of the cell juxtaposed to the nearest sinusoid, in the sheep, is strongly suggestive of a similar process. The facilitation of exocytosis preferentially at one border of a cell, may be assisted by the existence of a preassembled exocytotic fusion pore (Almers and Tse 1990). Clearly, a polarised distribution of fusion pores would facilitate highly directionalised exocytosis.

For exocytosis to occur, the secretory granule lumen must communicate with the extracellular environment. It is thought that, initially, early fusion pores open abruptly and may then expand allowing exocytosis, or simply close again (Nanavati *et al* 1992). Pore expansion is not effected by swelling of the granule matrix (Monck *et al* 1991) but may be driven by the tension contained within the granule membrane (Monck *et al* 1990; Oberhauser and Fernandez 1993). The rate constant of fusion pore formation is strictly temperature-dependent whilst pore closure exhibits a discontinuous relationship: closure is only temperature-dependant below 13^0C. It has been suggested that this is consistent with the opening phase requiring a molecular conformational change with a high activation energy, closure being effected by lipids which phase-separate at 13^0C (Oberhauser *et al* 1992).

Early ultrastructural observations demonstrated the presence of fine 'connecting strands' linking the granule membrane to the plasma membrane in the cells of the adrenal medulla in the golden hamster (Benedeczky and Smith 1972; review: Normann 1976). More recent studies in mast cells suggest that this structure is a protein scaffold, responsible for bringing the granule and plasma membranes into close apposition prior to membrane fusion (Fernandez *et al* 1992). Spontaneous membane fusion and exocytosis then results. Interaction of the granule membrane with the protein scaffold prior to membrane fusion may be mediated by rat brain 3a (Rab 3a) proteins which have been localised preferentially on the cytoplasmic surface of zymogen granules from pancreatic acinar cells (Jamieson *et al* 1992). The interaction of the Rab 3a protein with its effector in the protein scaffold leads to membrane fusion (Fernandez *et al* 1992). Although Rab 3 proteins may interact with the protein scaffold, they apparently do not associate with the cell membrane. During the exocytosis of zymogen granules from the apical plasma membrane of pancreatic acinar cells Rab 3-like protein was located on the zymogen granule membrane prior to exocytosis. Following the cessation of exocytosis Rab 3-like activity, within small vesicles, recycles to the trans Golgi network (TGN) without apparent association with the plasma membrane (Jena *et al* 1994). The authors suggest that this recycling involves a membrane dissociation-association cycle that

occurs during regulated exocytosis. The requirement of an interaction between Rab 3 protein on the granule membrane and a receptor in the vicinity of the plasma membrane to allow exocytosis, is supported by a recent study which demonstrated that a synthetic peptide related to the effector domain of Rab 3 inhibited LH exocytosis *in vitro* (Davidson *et al* 1993).

A further component of the protein scaffold may be the class of proteins known as the annexins. In the chromaffin cells of the adrenal medulla, synexin (annexin VII) promotes granule aggregation (Creutz *et al* 1978). This aggregation is calcium dependent (Creutz and Sterner 1983) with synexins associating with granule membranes. Final membrane contact may be achieved by the interaction of two synexin molecules which are in turn associated with their respective membranes (Zaks and Creutz 1991). In this way, members of the annexin family localised on the cell membrane could mediate the fusion of the secretory granule. Annexin II has been immunolocalised in close proximity to the attachment sites of chromaffin granules on the inner leaflet of the plasma membrane (Nakata *et al* 1990) and is believed to mediate calcium-dependent exocytosis (Ali *et al* 1989).

The localisation of the fusion apparatus, outlined above, only at the vascular membrane of the gonadotroph is the means by which directional exocytosis is achieved. It is possible that these components are only present in greater amounts once the gonadotroph becomes polarised thus preventing major exocytosis before the LH stores are suitably localised. Alternatively, fusion apparatus may be present throughout the oestrous cycle, granule transport occurring towards one aspect of the cell because membrane fusion there is an energetically favourable process. At this time, no studies on these proteins/structures in gonadotrophs have been reported. It is suggested that close examination of the subplasmalemmal regions of the gonadotroph cell, together with the regulation of the exocytotic machinery may provide a more exact explanation of the polarisation mechanism.

The mechanism by which cells polarise irregularly is unclear. It seems unlikely that the granule body is actually translocated to an abnormal intracellular location, given that a vascular signal appears involved. Two more tenable possibilities involve failure of cytoskeletal stabilisation and a lack of proteins composing the fusion pore and associated structures. If the calmodulin-mediated structural stabilisation of microfilaments was incomplete, as seems likely when either oestradiol or GnRH are absent, this would remove the framework along which granules may be transported to the cell surface. The exocytotic stimulus would only be able to release the granules that were transportable, the granules situated in the unstabilised area of the cytoplasm

remaining stationary. In this case, the granules left in the cytoplasm would give the cell a polarised appearance. This hypothesis would also explain, if correct, the disordered granule distributions observed in some instances where areas of the cytoplasm were agranular. It is also plausible that annexin proteins, which are associated with exocytotic sites at the plasma membrane, may require induction and act as the targeting signal for the granule membrane-cell membrane fusion process, before exocytosis can take place. Thus a failure of complete induction, leading to a lowered complement of these proteins at a specific area of the plasma membrane could prevent exocytosis in one aspect of the cell, again leading to the appearance of an irregular polarised gonadotroph.

The actual mechanism by which granules apparently move in one direction in the gonadotroph is not yet known. In the rat, although granule exocytosis occurs predominantly from the vascular border of the gonadotroph, granules are located around the entire cell periphery in the GnRH priming response (see Chapter 4). In this case, a unidirectional translocation is not required and it is possible to explain the observed distribution of exocytotic figures in terms of secretion being more energetically favourable in that region of the cell. The absence of fusion pores, in regions of the cell membranes except that abutting a blood vessel, may allow granules to be randomly transported around the cell periphery until a successful interaction with the membrane occurs. This may be due to the presence of a receptor for a granule membrane protein. Exocytosis would then result. In the sheep, the movement of the entire granule body in a single direction suggests, but does not preclude the possibility, that such a random interaction with the exocytotic membrane may not occur. The interaction between the granule and the microfilament is likely to be of critical importance in deciding the direction of translocation. No data is currently available concerning the nature of this interaction. Since high calcium concentrations may lead to the stabilisation of microfilaments it is unlikely that granules are transported via a process of assembly and disassembly occurring at either end of the filament. For the granule to control the formation of bonds between itself and the filament, a specialised complement of granule membrane enzymes and ligands may be required. In this case, analysis of the LH granule membrane may allow a greater understanding of the mechanisms which underlie the polarisation process. Such studies have been conducted on the chromaffin granule (Winkler 1993).

CHAPTER 7

THE ROLE OF THE GRANINS IN THE CONTROL OF GONADOTROPHIN SYNTHESIS AND RELEASE DURING THE OESTRUS CYCLE

Introduction

The granins are a family of acidic proteins with a ubiquitous distribution throughout the neuroendocrine system. Chromogranins, as the proteins were initially termed, were first isolated from bovine chromaffin granules around 25 years ago (Helle 1966; Blaschko *et al* 1967). Chromogranin A (CgA) was identified as the major constituent (Schneider *et al* 1967). To date, 6 members of the granin family have been identified: CgA; chromogranin B (CgB); secretogranin II (SgII); secretogranin III; secretogranin IV; secretogranin V and secretogranin VI (classification after Huttner *et al* 1991). Localisation of CgA, CgB and SgII in the ovine gonadotroph have been demonstrated histologically using immunohistochemistry (Rundle *et al* 1986). To date, no ultrastructural studies have been undertaken in the sheep. The co-localisation of SgII within the same secretory granule as LH in the rat gonadotroph has been shown (Watanabe *et al* 1991; Watanabe *et al* 1993) whilst GnRH treatment of human CgA-positive pituitary adenomas, *in vitro*, produces a preferential 2-fold increase in CgB mRNA (Song *et al* 1990). Furthermore, a down-regulation of SgII mRNA at behavioural oestrus has been reported in the rat (Anouar and Duval 1991). SgII is post-translationally processed to yield a 33 amino acid C-terminal fragment known as secretoneurin (Sn) (Vaudry and Conlon 1991, Kirchmair *et al* 1993, Marksteiner *et al* 1993). Studies on rat striatal slices have shown that Sn causes dopamine release *in vitro* (Saria *et al* 1993).

Whilst no function has yet been assigned to these proteins in the pituitary gland, their localisation within gonadotrophs and the apparent regulation of their gene expression by hormones significant in the oestrous cycle are suggestive of some involvement in the reproductive process. The aim of the following studies was to determine the regulation and localisation of CgA, CgB and SgII gene expression throughout the ovine oestrous cycle and the hormonal basis of any regulation observed.

7.1. Materials and Methods

7.1.1. Animals and Experimental Design

Pituitary glands from Welsh mountain ewes were used in the following studies. The tissue used was taken from experiments described in Chapters 4, 5 and 6. Briefly, this provided pituitary tissue from day 10 luteal phase, early follicular phase (PG+18h), oestrus, oestrus+9h, GnRH agonist-induced LH surge, oestrus+24h, GnRH agonist-induced LH surge (B)+24h, B+48h, B+72h and B+96h. This allowed the determination of granin expression and localisation during the period up to and including the preovulatory LH surge together with the refill phase where the LH stores are replenished following the surge. Pituitaries from animals treated with GnRH antagonist, oestradiol, GnRH antagonist plus oestradiol and anti oestradiol antibodies were also examined to determine the basis the hormonal regulation of granin mRNA.

7.1.2. Blood Sampling and Radioimmunoassay

Blood samples were taken as described in Chapters 4, 5 and 6 and plasma LH measured by the radioimmunoassay previously described (**2.2.3.1.**) to determine the endocrine status of each animal. Pituitary LH content and GnRH-binding activity were also determined as previously described (Chapters 4, 5 and 6). Immunoneutralisation of oestradiol was confirmed by binding of tracer (**2.2.3.3.**).

7.1.3. Immunocytochemistry and Determination of mRNA Abundance

CgA, CgB, SgII and Sn were localised on ultrathin sections cut from tissue which had been fixed and processed during the previous experiments (Chapters 4, 5 and 6). Ultrastructural localisation of the granins was achieved under the electron microscope by the immunogold technique (**2.2.6.3.**(b)). CgA antibody used for the immunolocalisation studies was a polyclonal antiserum raised against bovine CgA (Incstar Corporation, Stillwater, Minnesota, USA). The CgB (anti bovine CgB - raised against full length bovine CgB) and Sn (anti rat Sn - raised against the C-terminal 33 amino acids of rat SgII) antisera were kind gifts from Dr R. Fischer-Colbrie (Kirchmair *et al* 1993, Fischer-Colbrie and Frischenschlager 1985). The SgII polyclonal antiserum was raised against the N-terminal 17 amino acids of rat SgII (Pel-Freez, North Arkansas, Rogers, Arkansas). All antibodies were used at a dilution of 1:100 in THB and staining carried out using the immunogold localisation method previously described

(**2.2.6.3.**(b)). Co-localisation with LHβ subunit was achieved using a double immunogold method, different antigens being immunostained with different sized gold particles from opposing sides of the sections.

To determine if post-translational processing of SgII was modulated throughout the oestrous cycle, immunogold staining densities of SgII and Sn were compared in tissue sections taken from pituitaries in luteal phase, early follicular phase, oestrus+9h and GnRH agonist induced LH surge. Five gonadotrophs in each of the five animal per group were located via a systematic random sampling technique (**2.2.6.4.**). The number of gold particles per μm^2 was calculated from two randomised electron micrographs taken at 60000X magnification, per cell for Sn (5nm gold particles) and SgII (15nm gold particles). Mean gold particle densities were calculated for each time during the oestrous cycle. Cells were also assigned to class intervals, defined as 10% decrements of the maximum observed cell particle density. Frequency histograms were constructed for the percentage of cells in each class interval, thus allowing a comparison of the distribution of cellular staining densities between the time points under investigation.

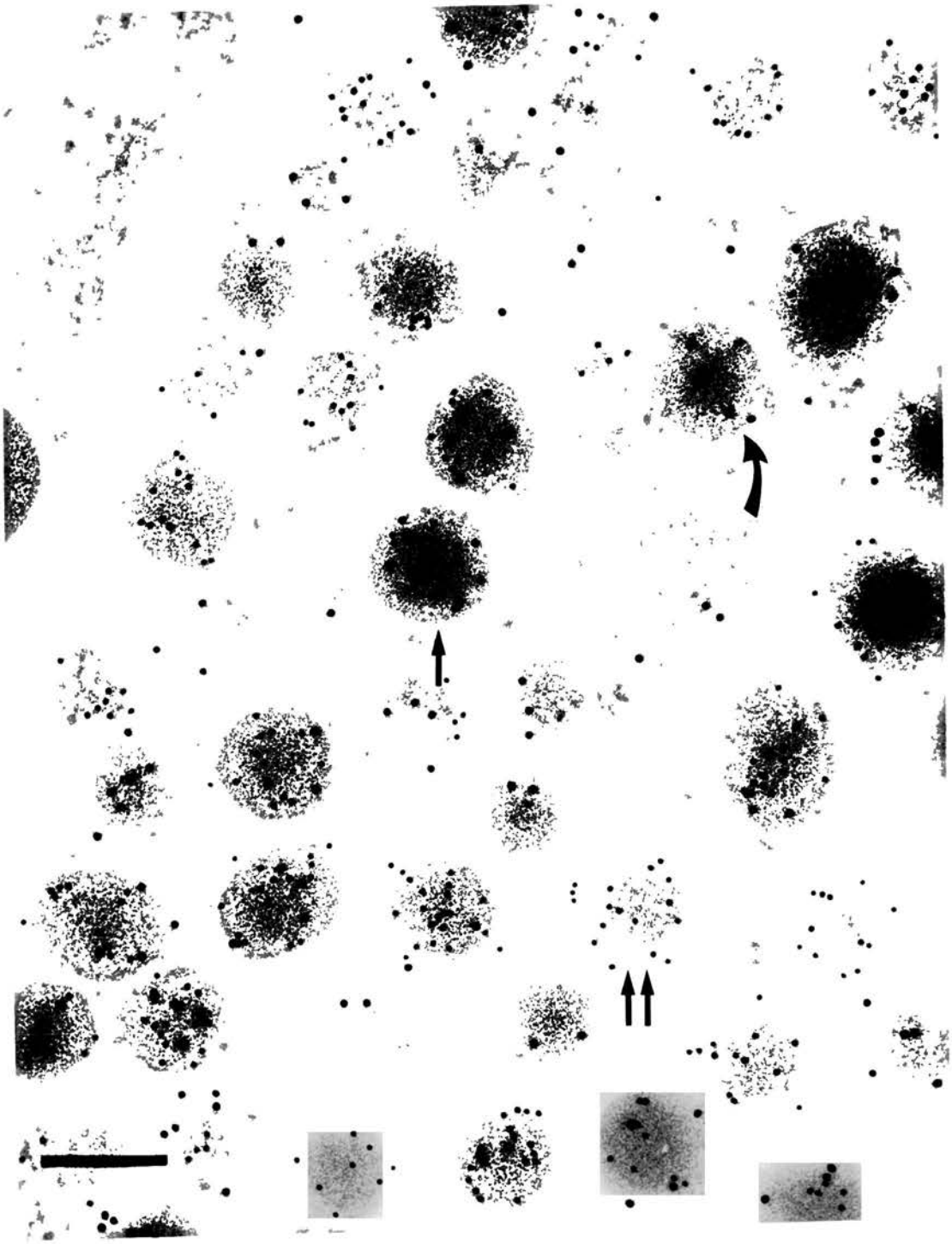
The pituitary glands from which total RNA had been extracted (Chapters 4, 5 and 6) was subjected to Northern analysis using cDNA probes. The CgB and SgII cDNA probes were kind gifts from Dr. R. Fischer-Colbrie. The SgII cDNA was constructed from a riboprobe inserted into a pGEM-57f(+) vector using the PCR/probe purification technique previously described (**2.2.7.**). The cDNA for CgA was kindly donated by Dr. A. Iacangelo. Levels of probe hybridisation were quantified using a Phosphor Imager (Molecular Dynamics).

7.2. Results

Radioimmunoassay of LH confirmed the timing of tissue collection whilst determination of oestradiol-binding capacity confirmed the ability of the plasma samples to immunoneutralise oestradiol (Chapters 4, 5 and 6).

SgII was co-localised with LHβ on ultrathin sections of adenohypophyseal tissue (Fig. 77). SgII immunoreactivity, indicated by the 5nm gold particles, was present throughout the matrix of LHβ immunopositive electron dense secretory granules, (demonstrated with 15nm gold particles). Immunoreactivity was interspersed between LHβ subunit, with no signal visible in any other aspect of the cell.

Figure 77. This transmission electron micrograph shows the co-localisation of LHβ subunit, indicated with 15nm gold particles, and SgII, indicated with 5nm gold particles, using an immunogold double localisation method. Most secretory granules were immunopositive for both LHβ subunit and SgII (↟). Small numbers of granule profiles were immunopositive for SgII only (↟ ↟), whilst others only stained for LHβ ( ). No SgII immunoreactivity was observed outwith secretory granule profiles. Magnification = 101000X. Scale bar = 195nm.



Light dense bodies (LDBs - Chapter 4), occurring in the natural cycle and induced by oestradiol administration, were SgII immunonegative in all instances (Fig. 78). SgII immunoreactivity was present from luteal through follicular phase to the preovulatory LH surge. At oestrus+24h and B+24h (24h after a buserelin-induced LH surge), the agranular cytoplasm contained no SgII immunoreactivity. During early luteal phase when the gonadotroph replenishes the intracellular LH stores, at B+48h, the granules observed in the cytoplasm contained SgII immunoreactivity (Fig. 79). Immunoreactivity was also present in granules at B+72h and B+96h.

Secretoneurin (visualised with 5nm gold particles) was colocalised with SgII (indicated by 15nm gold particles) in electron-dense granules in cells structurally typical of gonadotrophs (Fig. 80). Secretoneurin was located throughout the entire granule matrix with no specific localisation within the granule apparent and was present at every time point examined, with the exception of oestrus plus 24h and 24h after an induced LH surge. Immunoreactivity was absent in the agranular cytoplasm observed at these times.

CgA was localised within secretory granules of cells structurally typical of gonadotrophs. Using 10nm gold particles, CgA (Fig. 81) was located preferentially around the periphery of granules. Occasionally, immunoreactivity appeared to reside on the granule membrane. In isolated instances, low density staining was observed throughout the granule matrix. CgA was also present at all stages of the cycle, contained within granules, with its absence at oestrus+24h and B+24h. CgA staining was absent from the LDBs.

The distribution of CgB lay between that of SgII and CgA. Immunoreactivity was located peripherally, but not always in the absolute extremity of granules (Fig. 82). Immunostaining throughout the entire granule matrix was also observed. Staining was present at the same times throughout the oestrous cycle as the other granins studied. It was again absent at oestrus+24h and B+24h. Again, CgB immunoreactivity was absent from LDBs.

CgA, CgB, SgII and Sn staining in pituitary cell types other than gonadotrophs approached background levels.

Mean immunogold particle staining densities of SgII and Sn did not change significantly from luteal phase through to the LH surge (Fig. 83). When individual staining densities

Figure 78. Immunoreactivity for LHβ subunit, indicated by the 15nm gold particles, was localised within light dense bodies (LDB). SgII immunoreactivity, indicated by the 5nm gold particles, was present in secretory granules (↟) but absent from light dense bodies. Magnification = 119000X. Scale bar = 170nm.

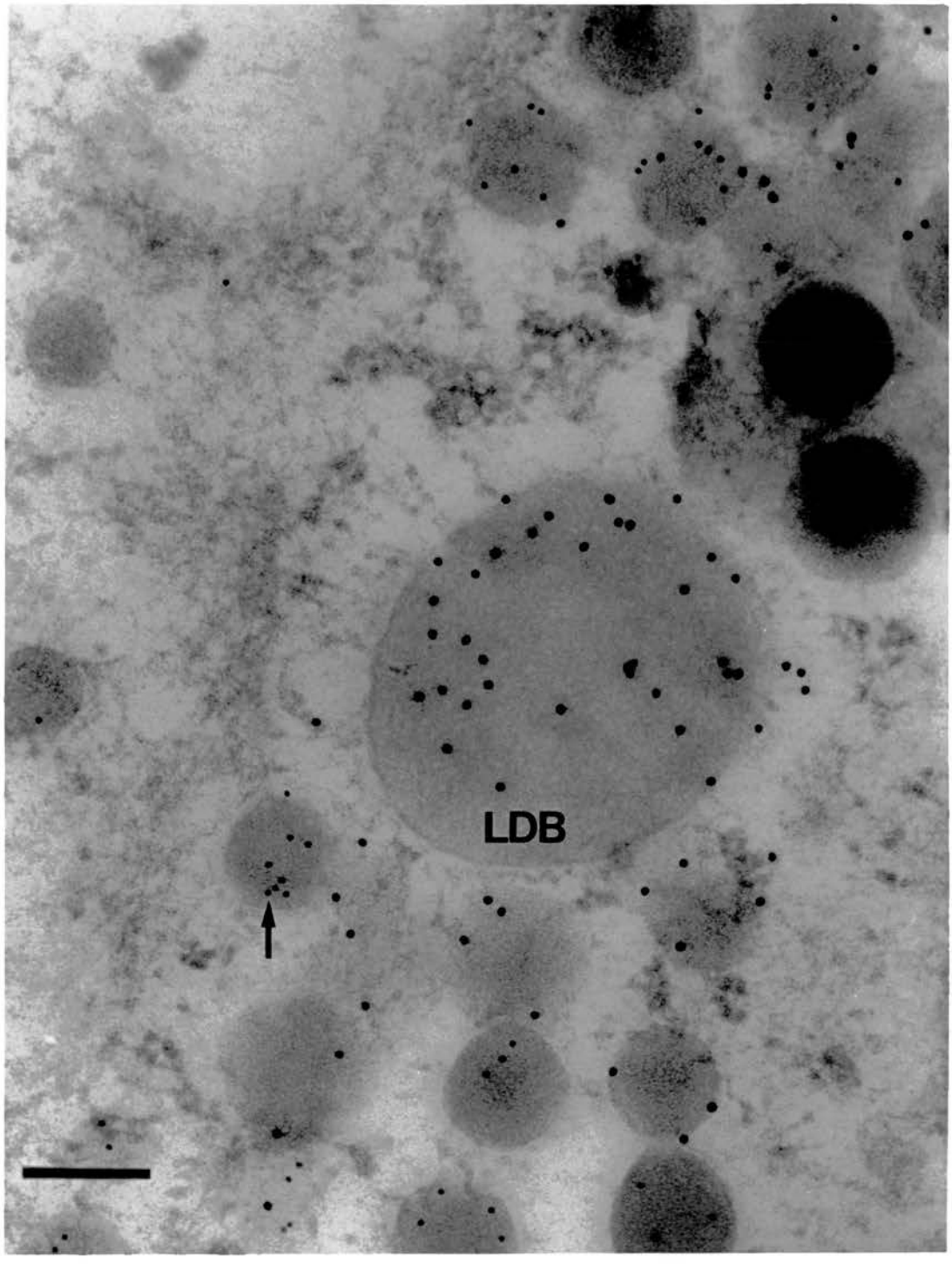

LDB

Figure 79. SgII, indicated with 10nm gold particles, was localised in the small secretory granules present in gonadotrophs 48h after the induction of an LH surge with the GnRH agonist buserelin. Immunoreactivity for SgII was detected solely in secretory granules and not in any other aspect of the cytosol. Levels in other pituitary cell types were indistinguishable from background staining. Magnification = 260000X. Scale bar = 80nm.

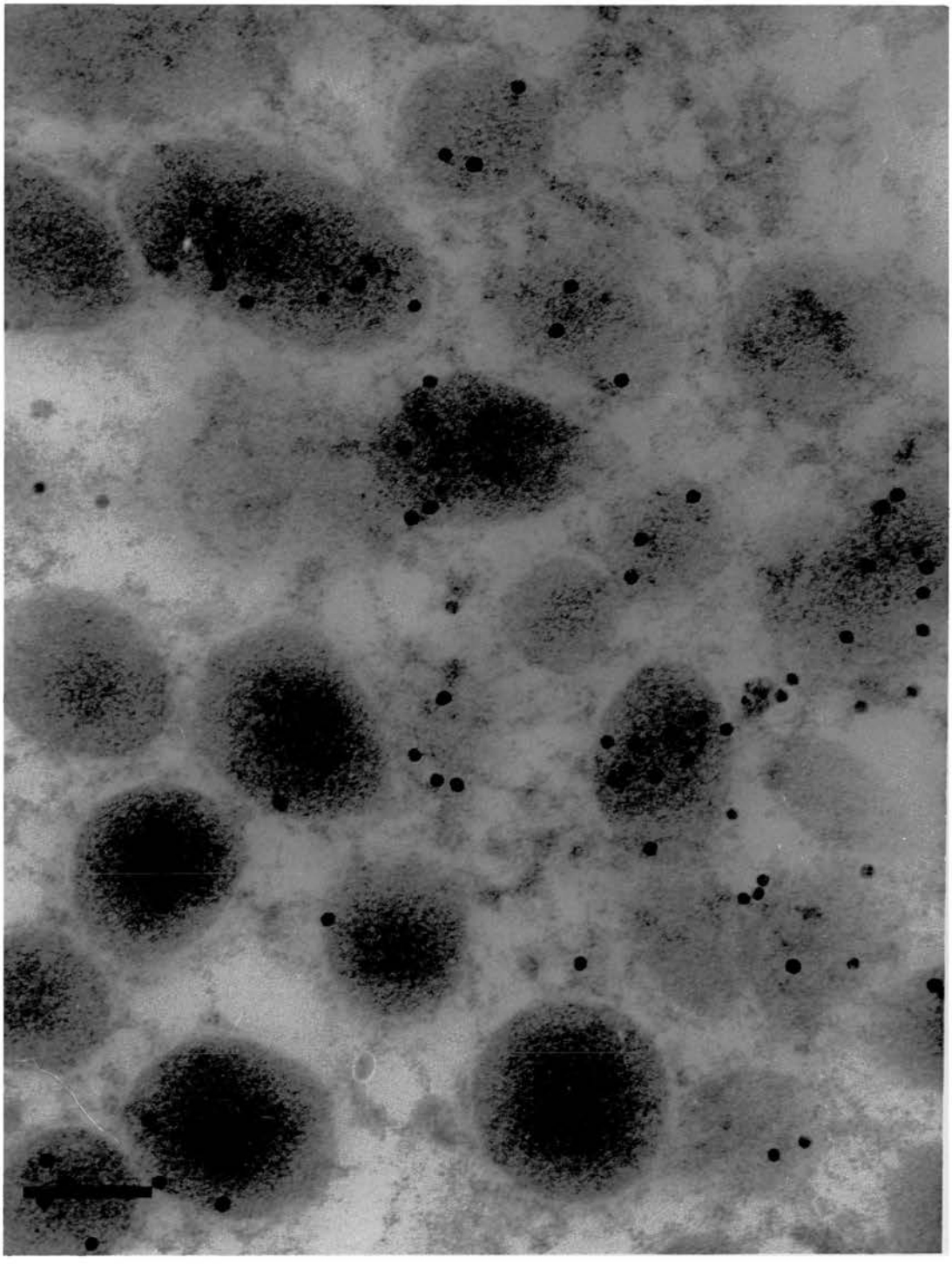


Figure 80. This transmission electron micrograph demonstrates the co-localisation of SgII and Sn using the immunogold double localisation method. Immunoreactivity for SgII, indicated by the 15nm gold particles, and Sn, indicated by the 5nm gold particles, was distributed throughout the matrix of most secretory granule profiles (↟). Some profiles displayed only SgII immunoreactivity (Sg) whilst others stained for Sn (S) only. Staining of Sn was only obtained over secretory granule profiles. Magnification = 119000X. Scale bar = 170nm.

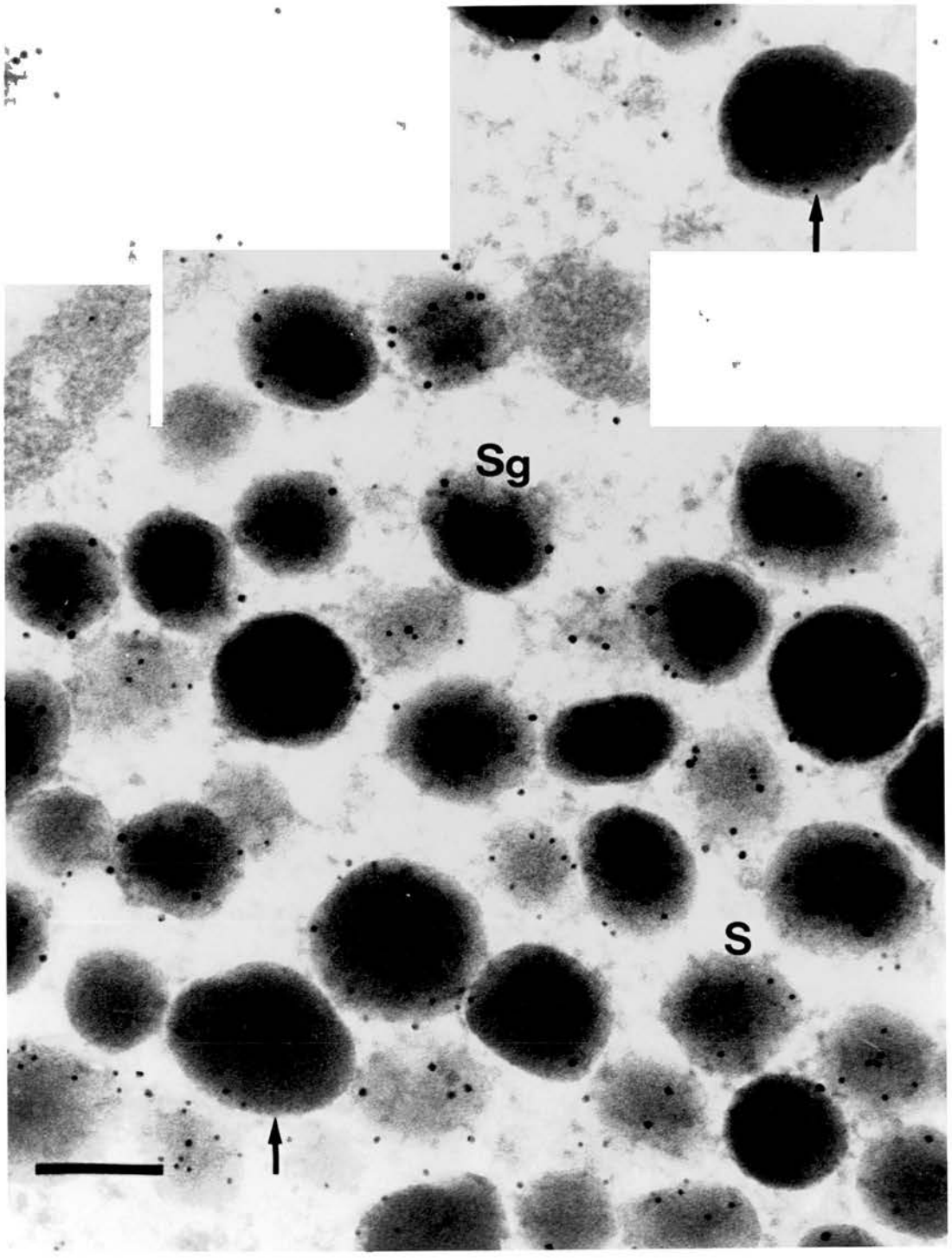
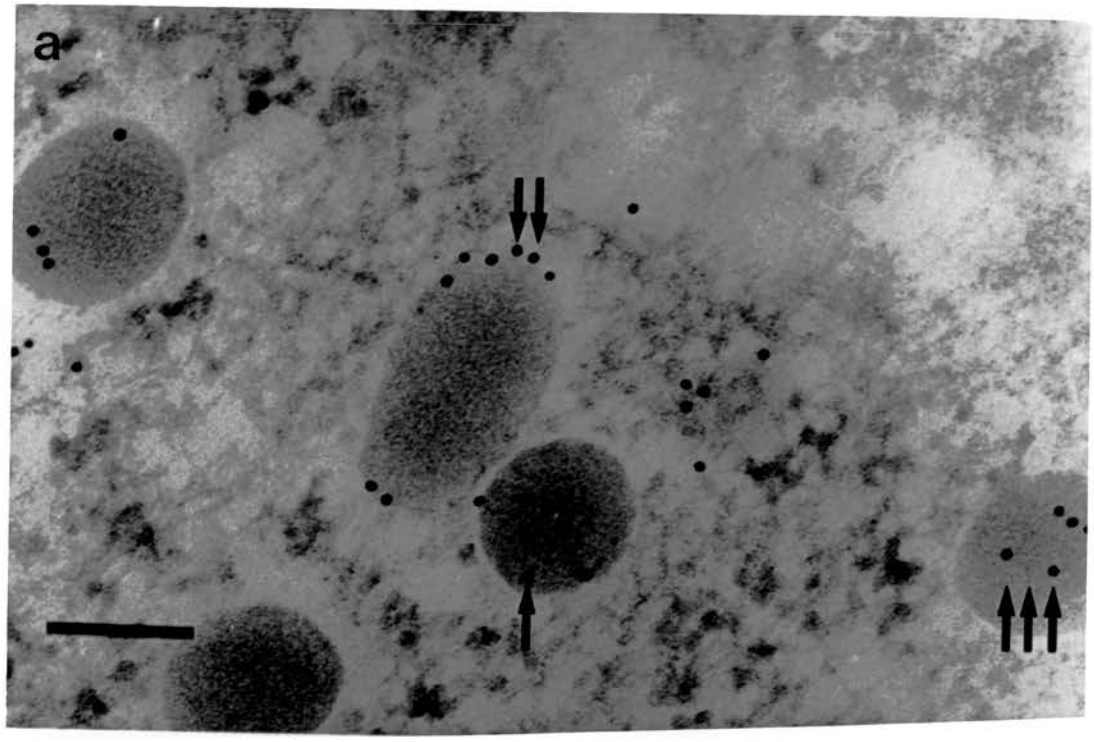

Sg
S

Figure 81. Transmission electron micrographs showing the subcellular localisation of CgA, indicated by the 10nm gold particles. Immunoreactivity for CgA was located preferentially in the periphery of gonadotroph secretory granules (↟) (a and b). On occasion, CgA appeared to be associated with the granule membrane (↟ ↟) (a). Rarely, CgA was located in the granule matrix core (↟ ↟ ↟) (a). No staining for CgA was found in the gonadotroph cytoplasm whilst staining in other pituitary cell types approached background levels.

a. Magnification = 178000X. Scale bar = 115nm.

b. Magnification = 200000X. Scale bar = 100nm.


a

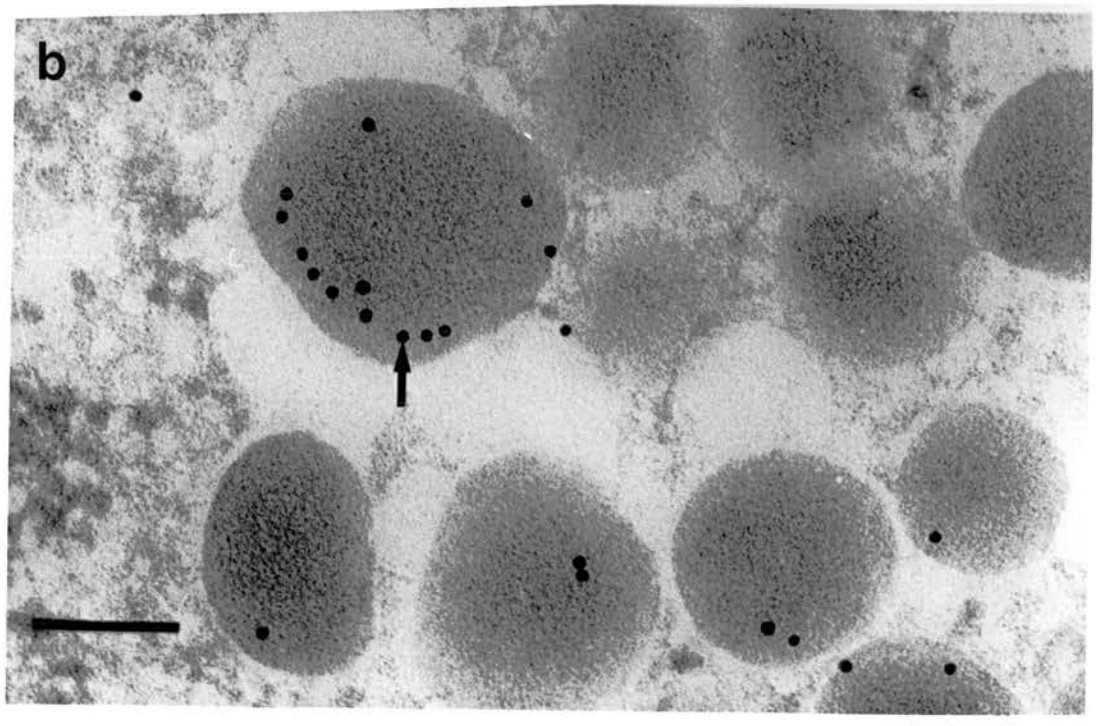
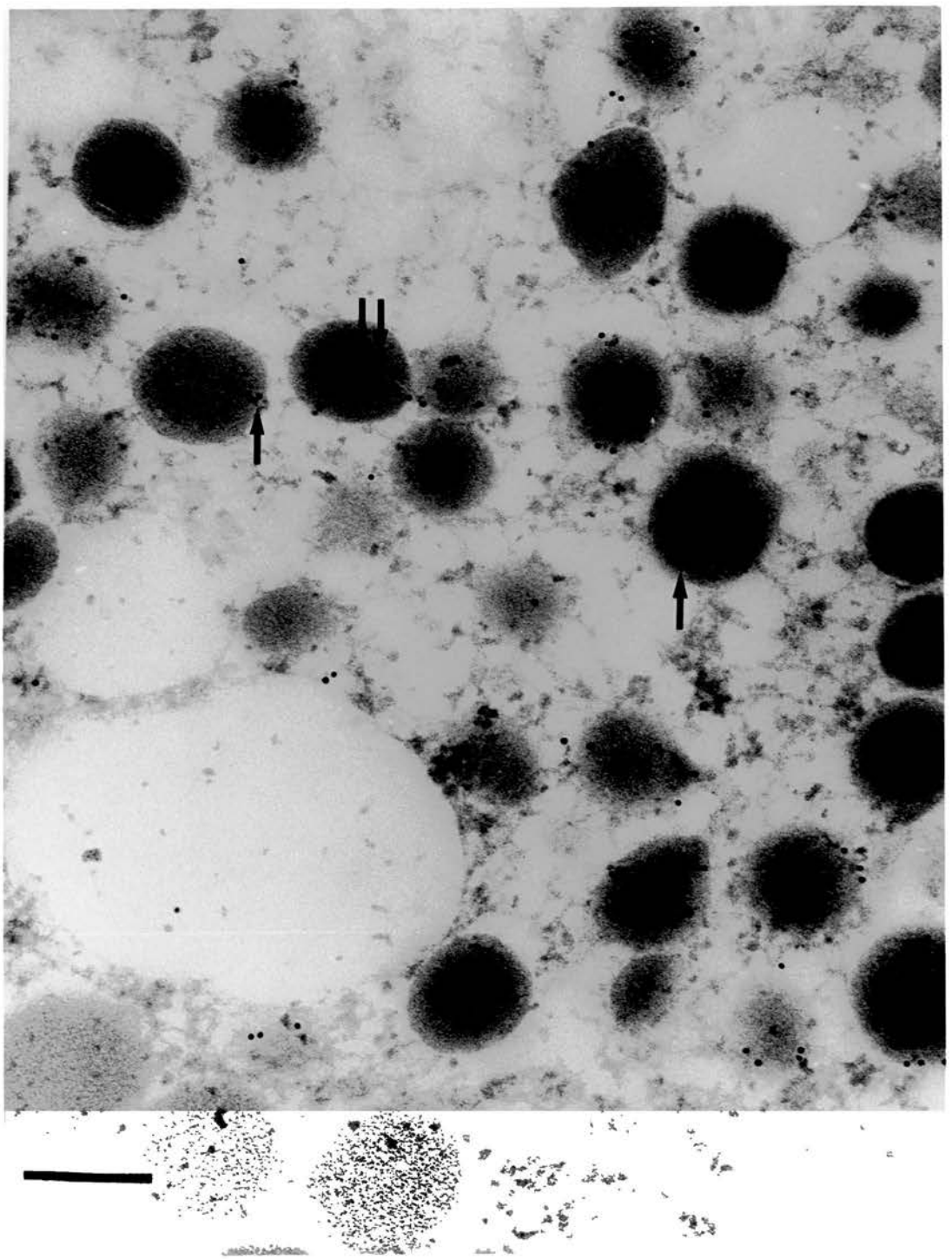

b

Figure 82. This transmission electron micrograph shows CgB (↑), identified with 10nm gold particles using the immunogold localisation technique, located in the periphery of secretory granules within the gonadotroph. Ocassionally, CgB immunoreactivity was observed in the granule core (↑ ↑). Staining for CgB was confined to secretory granule profiles in gonadotrophs and largely absent from the other pituitary cell types. Magnification = 101000X. Scale bar = 180nm.



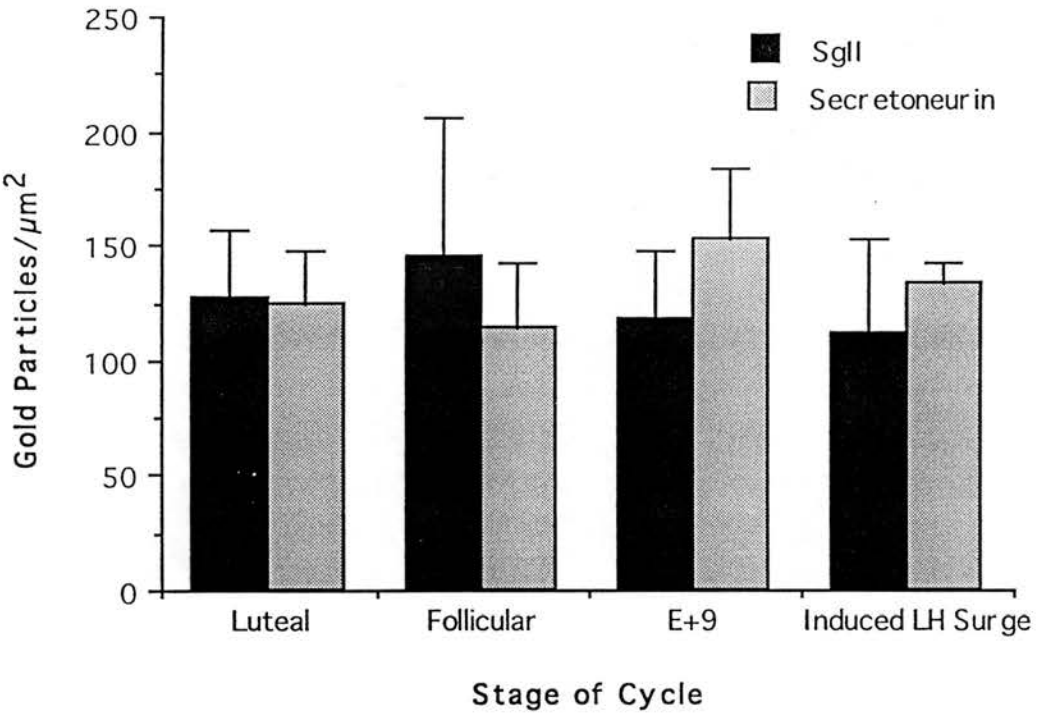


Figure 83. Gold particle staining densities (mean ± SEM) of SgII and Sn on day 12 of the luteal phase, early follicular phase 18h after prostaglandin-induced luteolysis, at oestrus plus 9h prior to the LH surge and during the LH surge. Gonadotrophs were selected by systematic random sampling and gold particle counts made on 5 cells in each of 5 animals (total n = 25) at each time point. Differences did not reach a level of significance (one-way ANOVA).

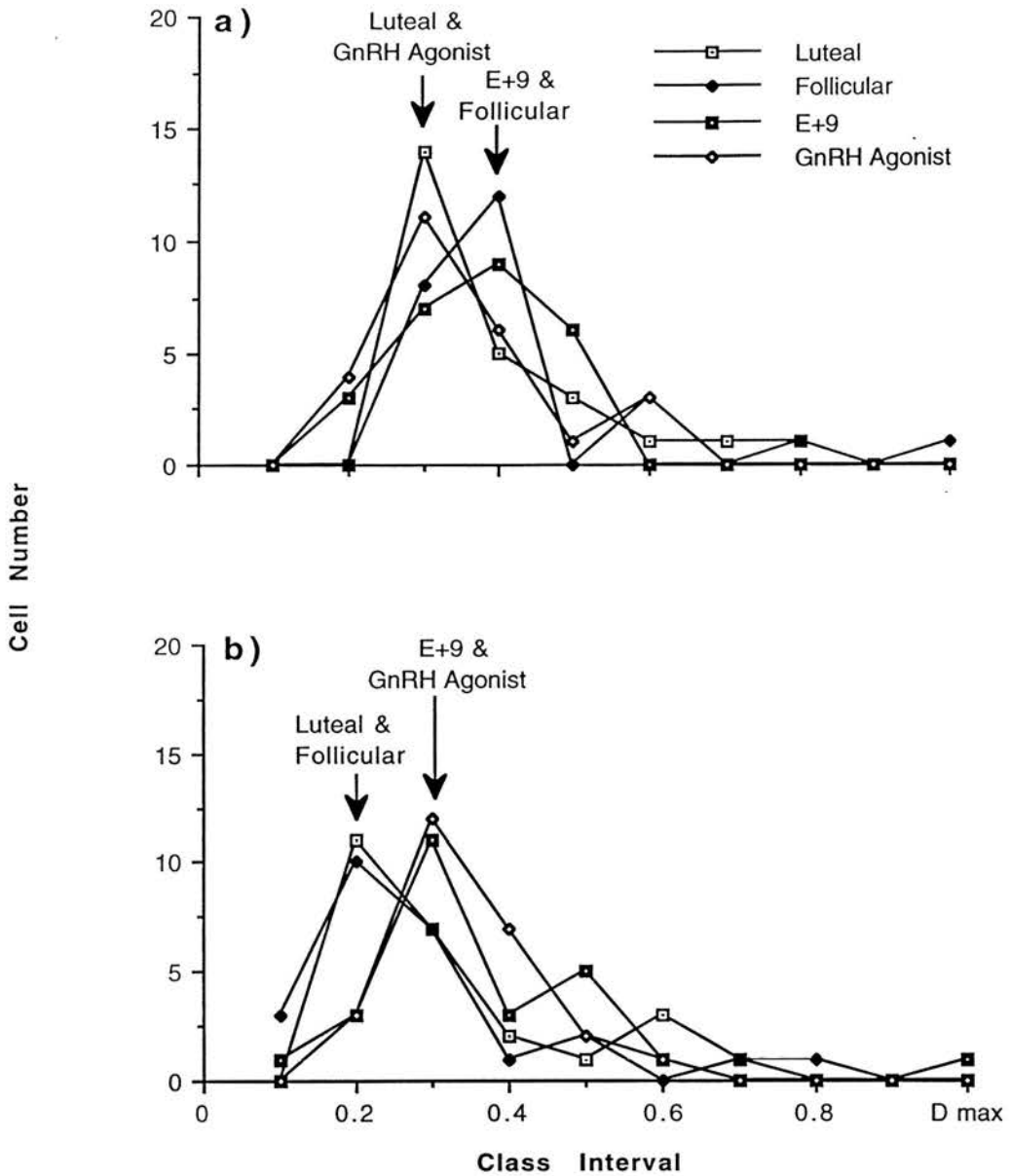


Figure 84. Frequency distribution of SgII and Sn staining density classes of gonadotrophs during luteal phase, early follicular phase, at oestrus plus 9h prior to the LH surge and during the LH surge. Gonadotrophs were selected by systematic random sampling and gold particle counts made on 5 cells in each of 5 animals (total n = 25) at each time point. The modal class for SgII (a) shifted from class 3 in luteal phase to class 4 in early follicular phase and at oestrus plus 9h prior to the LH surge. The modal class reverted back to class 3 during the induced LH surge. The modal class for Sn (b) shifted from class 2 in luteal and early follicular phase to class 3 prior to and during the LH surge.

within each cell were assigned to 10 class intervals expressed as percentages of the highest observed staining density, a shift in the modal class staining density of SgII was detected from luteal phase to the LH surge (Fig. 84a). The modal class increased from class 3 to class 4 from luteal to early follicular phase. The modal class reverted back to class 3 during the induced LH surge. Sn staining density displayed an increase in modal class from class 2 in luteal phase to class 3 during early follicular phase, oestrus plus 9h and the induced LH surge (Fig. 84b).

Gene expression studies demonstrated a significant ($P<0.05$) decrease of SgII mRNA from luteal through follicular phase to the preovulatory LH surge (Fig. 85a). Levels increased at oestrus+24h. In early luteal phase, during the replenishment of LH stores following the LH surge, steady state SgII mRNA abundance increased from B+24h to B+96h although this did not reach a level of significance (Fig. 85b).

CgA mRNA abundance decreased similarly from luteal through the follicular phase of the oestrous cycle (Fig. 86a). A non-significant increase was observed at oestrus+24h. Early luteal phase CgA mRNA levels showed no change from 24h to 96h after a GnRH agonist-induced LH surge (Fig. 86b). CgB mRNA abundance broadly paralleled the significant ($P<0.05$) decreases observed in the other granin mRNA species from luteal through follicular phase (Fig. 87a). No significant increase was evident at oestrus+24h. In common with CgA, no alteration in mRNA abundance occurred from B+24h to B+96h (Fig. 87b).

Treatment with GnRH antagonist, oestradiol, GnRH antagonist plus oestradiol and anti oestradiol produced no significant changes in the steady state mRNA abundance of SgII, CgA and CgB respectively (Fig. 88). Non-significant decreases were observed for SgII mRNA due to all treatments. Anti-oestradiol administration in the follicular phase appeared to increase CgA mRNA as did the GnRH antagonist whilst oestradiol treatment appeared to cause a decrease, although these changes were not statistically significant. CgB mRNA levels were unaffected with the exception of the oestradiol implant treatment which lead to a non-significant suppression.

7.3 Discussion

In the first ultrastructural immunocytochemical study of its kind in the sheep, CgA, CgB, SgII and Sn were localised within LHβ immunopositive secretory granules of

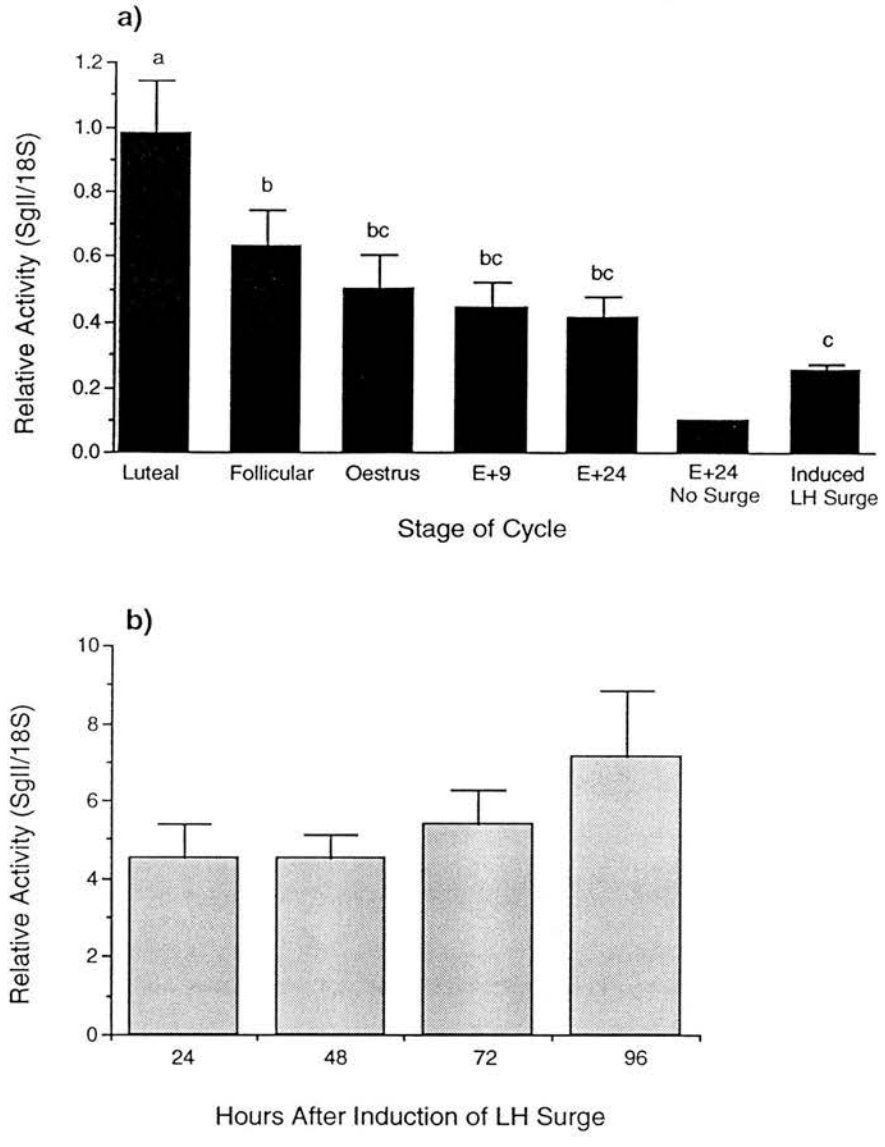


Figure 85. Changes in SgII mRNA abundance from a) day 12 luteal phase through follicular phase to oestrus plus 24h, after the LH surge and b) 24h, 48h, 72h and 96h after a buserelin induced LH surge. Changes in SgII mRNA abundance are expressed relative to 18S ribosomal mRNA subunit. Blots were exposed for 2h and the results quantified using a Phosphor Imager. Values are given as mean ± SEM, a) n=5 in all groups except oestrus plus 24h in which n=4 as one ewe failed to produce an LH surge, b) n=6. Data is shown as mean ± SEM and analysed by one way ANOVA. Different letters denote significant ($P<0.05$) differences. Each histogram is composed of an analysis from separate Northern blots.

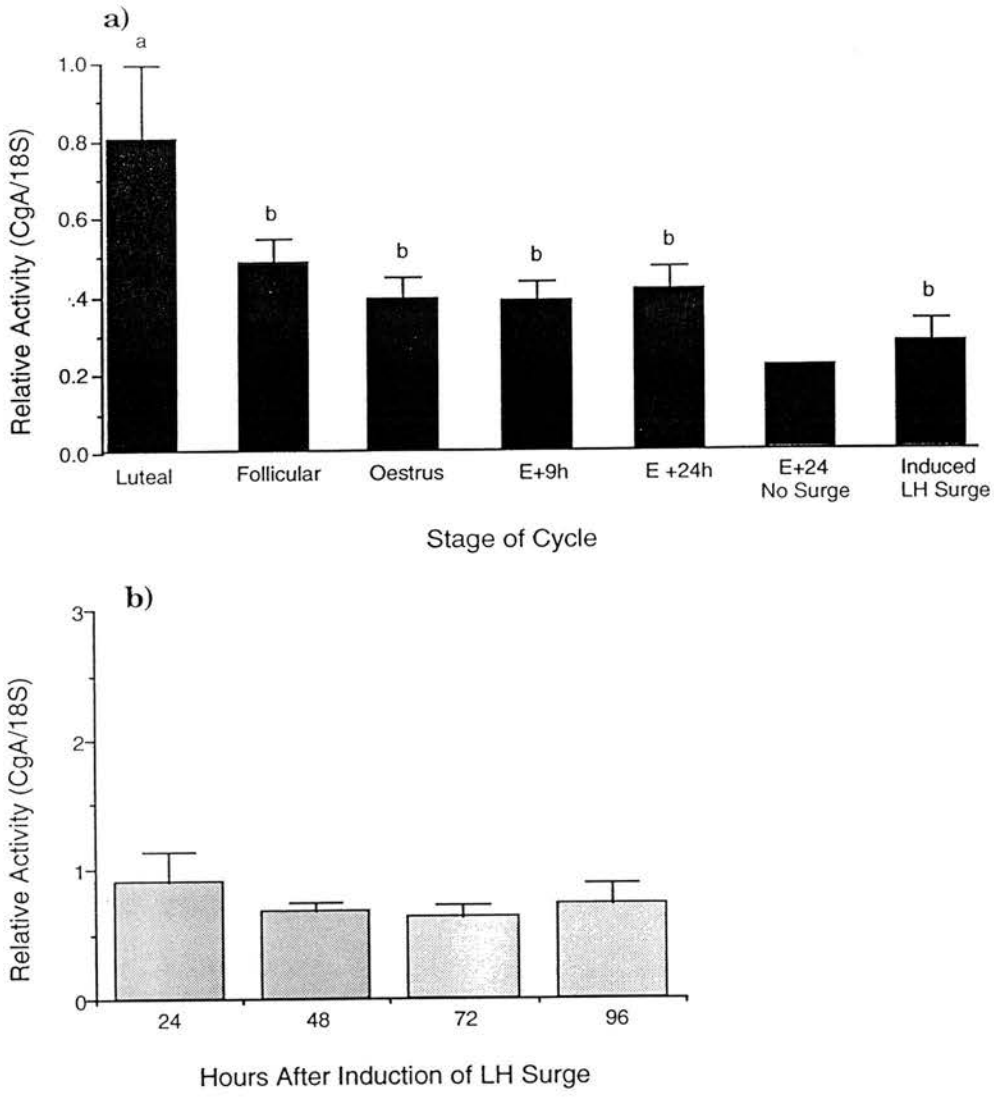


Figure 86. Changes in CgA mRNA abundance from a) day 12 luteal phase through follicular phase to oestrus plus 24h, after the LH surge and b) 24h, 48h, 72h and 96h after a buserelin induced LH surge. Changes in CgA mRNA abundance are expressed relative to 18S ribosomal mRNA subunit. Blots were exposed for 2-6h and the results quantified using a Phosphor Imager. Values are given as mean ± SEM, a) n=5 in all groups except oestrus plus 24h in which n=4 as one ewe failed to produce an LH surge, b) n=6. Data is shown as mean ± SEM and analysed by one-way ANOVA. Different letters denote significant ($P<0.05$) differences. Each histogram is composed of an analysis from separate Northern blots.

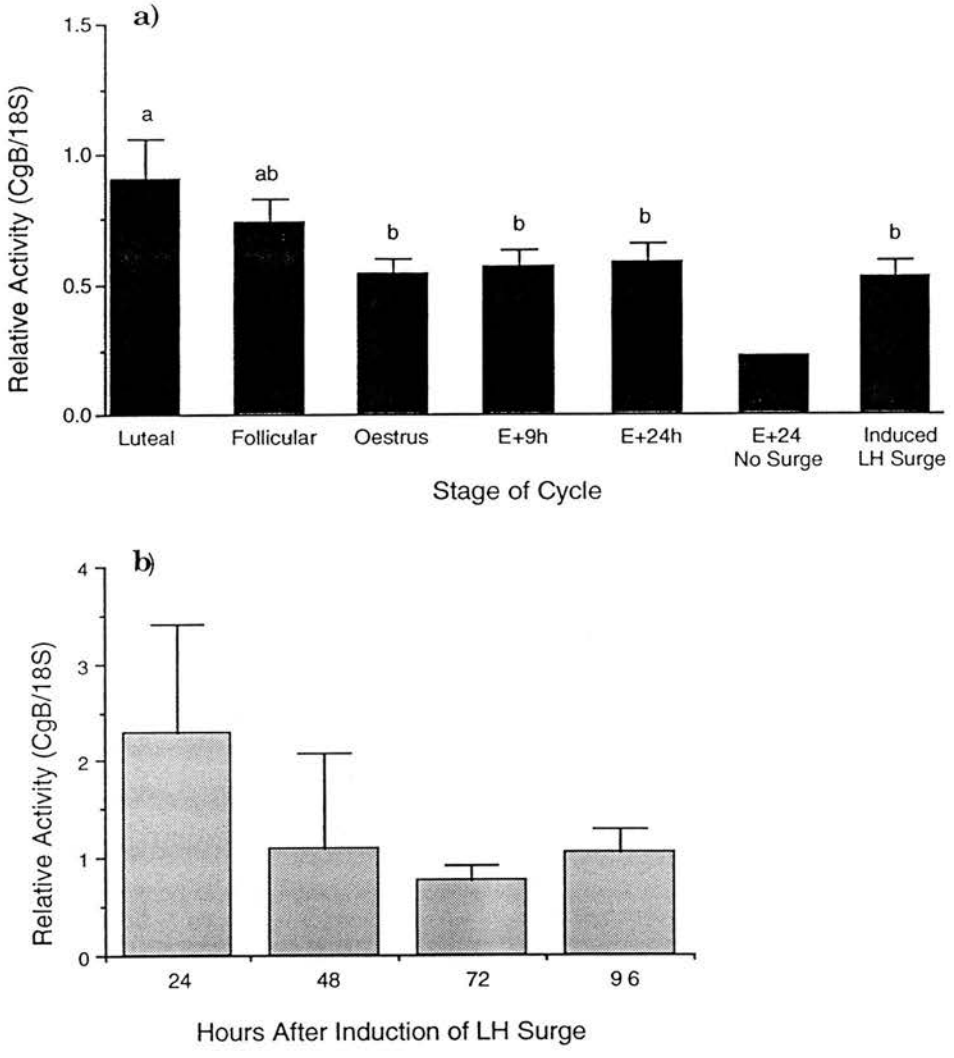


Figure 87. Changes in CgB mRNA abundance from a) day 12 luteal phase through follicular phase to oestrus plus 24h, after the LH surge and b) 24h, 48h, 72h and 96h after a buserelin induced LH surge. Changes in CgB mRNA abundance are expressed relative to 18S ribosomal mRNA subunit. Blots were exposed for 6-18h and the results quantified using a Phosphor Imager. Values are given as mean ± SEM, a) n=5 in all groups except oestrus plus 24h in which n=4 as one ewe failed to produce an LH surge, b) n=6. Data is shown as mean ± SEM and analysed by one-way ANOVA. Different letters denote significant ($P<0.05$) differences. Each histogram is composed of an analysis from separate Northern blots.

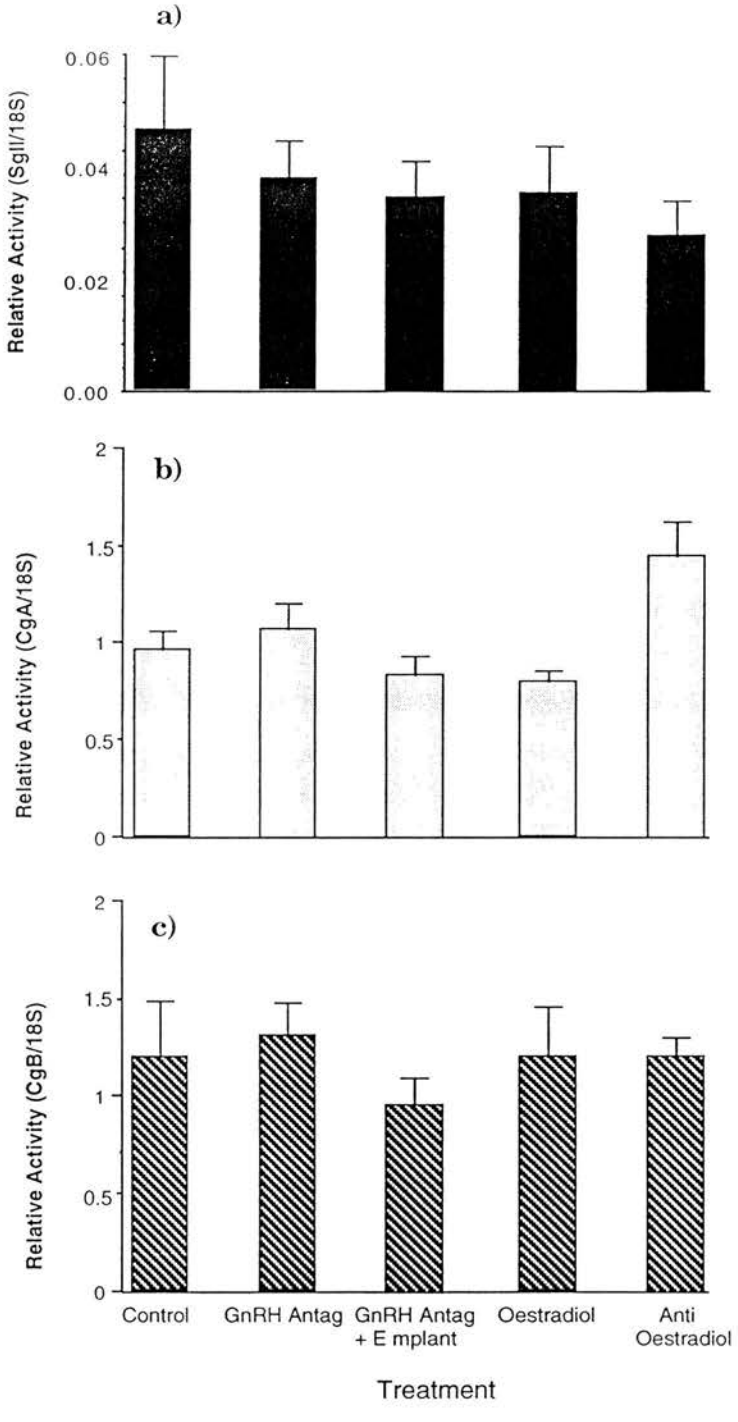


Figure 88. Changes in a) SgII, b) CgA and c) CgB mRNA abundance in follicular phase control ewes and ewes treated with GnRH antagonist, GnRH antagonist plus oestradiol, oestradiol and anti-oestradiol at the time of prostaglandin-induced luteolysis, 36h before death Results are mean ± SEM, n=6 per group. Treatments did not cause any significant alterations in SgII, CgA and CgB mRNA abundance (one-way ANOVA). Each histogram is composed of an analysis from separate Northern blots.

gonadotrophs in the adenohypophysis. These results demonstrate a similar distribution to that found in the bovine gonadotroph, where all secretory granules are immunopositive for CgA, CgB and SgII (Bassetti *et al* 1990). It was notable that LDBs were immunonegative for all three granins studied. This will be discussed further in Chapter 8.

Previous ultrastructural studies on the rat gonadotroph have demonstrated a dichotomy of distribution, within different size classes of secretory granule, of CgA and SgII. CgA was co-localised with LH and FSH in moderately electron dense granules of approximately 500nm diameter, whilst SgII co-localised with LH in electron dense granules of 200nm diameter (Watanabe *et al* 1991). The authors postulated that this distribution was indicative of two regulated secretory pathways and may therefore mediate the differential release of LH and FSH. No such distribution of staining was observed in the sheep. Since all granule profiles have been shown to be LHβ immunopositive (**4.5.**), it has been shown that the four granins investigated all co-localise with LH. If the granins do regulate differential gonadotrophin release in the rat, their role would appear to differ in the sheep.

It was notable that immunostaining of the granins was only achieved when granules were present. Although regions of LHβ immunoreactivity were observed at B+24h (24h after the induction of an LH surge) throughout the agranular cytoplasm (**5.2.**), no granin immunoreactivity was detected. It has previously been suggested that the granins may function in the regulation of the composition of the secretory granule matrix (Winkler *et al* 1986; Konecki *et al* 1987). In contrast, when intracellular conditions leading to condensation are duplicated, SgII aggregates and in the process excludes other secretory substances (Gerdes *et al* 1989). The pI of SgII (5.0-5.1) suggests that it could only function in the packaging of highly basic proteins (Conn *et al* 1992). The co-localisation with LHβ immunoreactivity within the same granule indicates that these biochemical incompatibilities are overcome in a physiological situation. Furthermore, the correlation of granin immunoreactivity with the appearance of granules suggests that these proteins may be essential structural components of the granule and that a lack of translation could prevent granule synthesis.

The subcellular localisation of the granins within the secretory granule differed from that reported in the rat. Watanabe *et al* (1991) showed CgA distributed throughout the entire granule matrix. This study has localised CgA preferentially towards the granule

membrane, in the periphery of the secretory granule. This is a similar localisation to that reported in the glucagon storage vesicles of the exocrine pancreas (Varndell *et al* 1985; Ehrhard *et al* 1986; Hearn 1987), secretory vesicles of Merkel cells (Hartschuh *et al* 1989) and intestinal secretin cells (Usellini *et al* 1990). The localisation of CgA to the granule membrane is pH-dependant, occurring at the intravesicular pH of 5.5, whilst the putative membrane binding domain is thought to lie within the N-terminal residues 18-37 (Yoo 1993a). The same author demonstrated the dissociation of CgA from membranes at the physiological pH of 7.5, a possible mechanism by which the protein is released upon granule fusion with the plasma membrane.

This peripheral localisation may be functionally significant with respect to exocytosis. When GnRH interacts with its receptor on the gonadotroph membrane, one of the principle events in the activation of the cell secretory response is an elevation of the intracellular calcium concentration (Clapper and Conn 1985; Leong *et al* 1986; Chang *et al* 1986; Turgeon and Waring 1986; McArdle *et al* 1992; Anderson *et al* 1992). CgA and CgB are known to bind calcium (Reiffen and Gratzl 1986a, b; Gorr *et al* 1989), whilst calcium has also been shown to increase the association of CgA with membranes (Gorr *et al* 1988; Leiser and Sherwood 1989). This calcium may be transmitted to CgA by the intraluminal loop domains of the inositol 1,4,5-trisphosphate receptor (Yoo and Lewis 1994). It may be that calcium bound within the secretory granule allows the peripherally located/membrane associated CgA to associate with the plasma membrane thus promoting membrane fusion and exocytosis. The fact that CgA was localised in some granules throughout the granule matrix suggests that the protein may migrate to the periphery of the granule when sufficient calcium is present. This migration may be part of the granule maturation process and required before granule membrane-plasma membrane fusion can take place. Alternatively, the distribution may be a consequence of varying degrees of post translational processing of CgA at different areas throughout the granule matrix. Cleavage of the protein, located in the perigranular region, into a number of smaller fragments may render it undetectable by the antibody used for immunohistochemistry, whilst a lack of processing in the granule core would result in the successful immunodetection of the protein. Furthermore, a membrane form of CgA may function as part of the protein scaffold (Chapter 6) which is thought to draw together lipid bilayers prior to their fusion, this function being activated only during the high intracellular calcium concentrations experienced when exocytosis is triggered. It is not clear, at present, if CgA cleavage products increase their association with membranes in the presence of calcium.

A further role may exist for CgA in the movement of the granule body during the polarisation process of the gonadotroph (Chapter 4). The protein has recently been shown to exist in dimer and tetramer forms (Yoo and Lewis 1992), the construction of which may be mediated by the C-terminal region via calcium (Yoo and Lewis 1993). The formation of CgA dimers and tetramers between secretory granules may result in the co-ordinated transport of the granule body. It is possible that polymeric associations between granules would allow the cytoskeleton to transport granules without having to bind individually to each one.

CgA appears to contain a dominant targeting signal for the regulated secretory pathway. The expression of chimeric proteins composed of full length or N terminal 226 amino acids truncations of CgA fused to chloramphenicol acetyltransferase (CAT), resulted in the diversion of CAT into regulated secretory vesicles in the rat pheochromocytoma PC-12 cell line (Parmer *et al* 1993). Although CgA composes only around 4.5% of an adenohypophyseal granule (Barbosa *et al* 1991), this may still be sufficient to act as a molecular chaperone targeting the LH subunits to the regulated secretory pathway.

The distribution of CgB observed within the granule is similar to that reported in somatomammotroph cells in the bovine anterior pituitary (Bassetti *et al* 1990), chromaffin cells and gastrin cells (Cetin and Grube 1991). Although CgB was localised in the granule periphery, immunoreactivity did not appear as closely related to the granule membrane as CgA. CgB has been shown to associate with granule membranes (Pimplikar and Huttner 1992) at pH 5.5 present within the granule matrix and disassociate at 7.5 thus allowing its release following the fusion of the granule and plasma membranes (Yoo 1993b). The association of CgB with the trans Golgi network (TGN) membranes is facilitated by the acidic intragranular pH in common with CgA (Yoo 1993b). No such localisation was demonstrated in sheep gonadotrophs. A lack of CgB staining in the TGN membranes during times of the oestrous cycle when *de novo* protein synthesis was at its lowest is compatible with the reduced level of synthetic activity. However, due to the lack of newly condensed granules budding off the Golgi membrane, the absence of signal during the luteal phase when synthesis appears maximal is indicative that the intact protein is probably not present. From the observed localisation, it would appear unlikely that CgB is involved in the initial membrane-membrane interaction prior to exocytosis.

Due to the existence of two cysteine residues in the vicinity of the amino-terminus, an intramolecular disulphide bond causes the formation of a 20 amino acid loop structure within the protein (Benedum *et al* 1987). In PC12 cells, the reduction of this bond with dithiothreitol (DTT) in the TGN led to the diversion of newly synthesised CgB into constitutive secretory vesicles and its immediate release from the cell (Chanat *et al* 1993). The authors suggested that the disulphide bond may be required for interaction with membrane associated CgB in the TGN during the sorting process. Due to the apparent absence of TGN membrane-bound CgB in the sheep gonadotroph, it may be that the conformation of CgB is important in the formation of the granule body. However, the same authors state that lysis of the constitutive vesicles of CgB (Chanat and Huttner 1991) in aggregative buffer still lead to CgB aggregation. It is possible that the disulphide bond and the associated tertiary protein structure are required for the aggregation and / or packaging with other secretory products that occurs *in vivo* , and that the removal of these compounds in the *in vitro* system allows successful aggregation without the disulphide bond.

Stimulated secretion *in vitro* in PC12 cells led to a fraction of the CgB remaining tightly associated with the plasma membrane following exocytosis (Pimplikar and Huttner 1992). Following a period of incubation, this form of CgB was subsequently internalised and degraded. Histologically, CgB activity has also been reported to be localised in the region of the plasma membrane (Watanabe *et al* 1991). This observation, together with the ability of CgB to bind calcium suggests a role in the process of exocytosis. Specifically, calcium induced CgB aggregation may act as a prelude to the protein functioning as a structural component of the fusion pore (Chapter 6), which forms during exocytosis to allow the connection of the intra and extra cellular environments. This may assist in the lengthening of the open time for the pore, either by contributing to the ultrastructure or by a slower rate of passage. This may result in a more efficient exocytosis of the major hormone components of the gonadotroph secretory granule. The endocytosis of the CgB following the secretory event means that a role as a targeting protein for membrane retrieval and recycling cannot be excluded. The lack of CgB immunoreactivity at the plasma membrane of ovine gonadotrophs may be due to two factors:

1. the length of time between tissue removal and fixation was such that acute and basal exocytosis had stopped, due to removal of the GnRH input, and all membrane associated CgB had been internalised.

2. membrane ultrastructure was consistently poor throughout the studies due to the omission of osmium tetroxide fixation, and the resulting antigen destruction may have rendered them undetectable by the antibodies used for immunocytochemistry.

Of these explanations, the first is more tenable due to the failure of a tannic acid fixation protocol to capture any exocytotic figures in gonadotrophs from tissue fixed after the same time interval post mortem (R. Currie, unpublished). Also, the detection of CgA immunoreactivity on the granule membrane suggests that, although the ultrastructure was perturbed, immunogenicity was sufficiently preserved to allow detection in the region of the membrane.

SgII appeared to stain with the greatest intensity of the three granins studied. The distribution throughout the granule matrix, but not the granule membrane, may be due to the differing physicochemical properties of SgII from CgA and CgB. The disulphide loop structure described above for CgB is also present in CgA (Benedum *et al* 1986; Iacangelo *et al* 1986). No such structure exists in the SgII molecule due to the absence of cysteine residues within its primary structure (Gerdes *et al* 1989). The absence of SgII at or in the vicinity of membranes, in this or any other study, suggests that any function the protein may have in the actual process of exocytosis would be substantially different to that of CgA and CgB. Whilst a contribution to fusion pore structure cannot be excluded, it would seem more probable that any structural function would be associated with the secretory granule.

The shifts in the distributions of cell staining densities of SgII and Sn from luteal phase through follicular phase to the preovulatory LH surge, indicate that SgII exists in higher concentrations in the larger granule preparations. The fact that the SgII modal class in mid LH surge is of a lower staining density than the early follicular and oestrus+9h groups is a likely consequence of the greater degree of post-translational processing of the protein that apparently occurs in a subpopulation of the larger granule size classes. Increased cleavage of SgII to form Sn, and a resultant change in the tertiary structure of the protein, may render the SgII remnant less recognisable to the antibody, thus resulting in a decreased staining density. The mean staining densities for luteal, early follicular, oestrus+9h and mid LH surge show no significant changes. For increased processing of SgII to occur in tandem with this observation, a further population of cells must possess decreased staining densities to allow the overall mean to remain unchanged. This is plausible if the model for the recruitment of gonadotrophs into the polarised state is reconsidered (Chapter 4). As waves of gonadotrophs

progressively polarise and empty, the cells which have been recruited from the non polarised cohort will contain greater numbers of the smaller granule populations. The decreased staining densities of these granule populations would counter balance the increased contribution from the larger granules. Theoretically, a time point should exist towards the end of the preovulatory LH surge with most gonadotrophs empty when only the largest granule sizes are present. A future experiment with tissue taken at this time point may be able to measure an increase in mean staining density of Sn and a corresponding decrease in the SgII mean value. This would demonstrate conclusively that SgII is processed to a greater degree in the largest granule size classes. It is thought that around 25% of the immunopositive staining achieved with the Sn antibody is a result of the cross-reaction with intact SgII (R. Fischer-Colbrie - personal communication).

Secretoneurin has been shown to release dopamine (Saria *et al* 1993). It is unclear what role an increased amount of this compound would have following the LH surge. The absence of any structure capable of dopamine release suggests that either Sn has a different action in the adenohypophysis and functions as a paracrine regulator of an, as yet, undefined cellular function, or that it has no endocrine role and the increased concentration of Sn in large granules is merely a consequence of the cleavage enzymes having a greater amount of SgII substrate. Recently five putative nuclear localisation sites, four of which coincided with dibasic residues previously thought to function as cleavage sites, have been identified on the SgII intact protein in the rat (Kakar *et al* 1993b). This is suggestive of a paracrine role in the control of gene transcription rates.

The predominant localisation of granin immunostaining in LHβ–positive cells indicated that any changes in mRNA abundance could be attributed to alterations of gene expression within the gonadotroph.

The significant reduction of mRNA levels of CgA, CgB and SgII, demonstrated from the luteal to follicular phase, paralleled the changes in the steady state abundance of LHβ mRNA. Oestradiol has been shown to down-regulate SgII mRNA in female rats whilst GnRH causes up regulation and an increase in SgII synthesis (Kakar *et al* 1993b; Anouar *et al* 1991a, b; Chanat *et al* 1986). It is of note that during the rat oestrous cycle, the highest levels of SgII mRNA have been described at oestrus (Kakar *et al* 1993b), whilst another group indicate the lowest levels at oestrus with a peak value at di-oestrus II (Anouar and Duval 1991). As both groups observed SgII mRNA inhibition by oestradiol, this discrepancy remains unexplained at present and requires further

study. Oestradiol administration to male rats leads to up regulation of SgII mRNA (Fischer-Colbrie *et al* 1992) The down-regulation observed in this experiment from the luteal phase throughout the follicular phase to the LH surge suggests that, *in vivo*, oestradiol is the dominant modulator of SgII mRNA abundance. In the ewe, the negative effects of oestradiol apparently take precedence over the potentially stimulatory influence of increasing GnRH pulse frequency which occurs through follicular phase as the LH surge approaches. In the initial four days post LH surge, SgII mRNA appeared to increase although the trend just failed to reach a level of significance. The low plasma concentrations of oestradiol at this time during the cycle (Campbell *et al* 1990) should allow mRNA levels to increase back to the values observed at day 10 luteal phase. The use of GnRH antagonist individually and in combination with oestradiol implants paralleled the results obtained in the rat for the roles of GnRH and oestradiol in the control of SgII gene expression, although none of the effects were statistically significant. The non-significant suppression of SgII with anti-oestradiol was unexpected. The removal of the suppressive effects of oestradiol by immunoneutralisation would be expected to lead to mRNA up regulation due to the stimulatory effects of GnRH. However, a similar suppressive effect occurred on LHβ mRNA in the same treatment group.

Although the presence of a second, smaller 1.7kb SgII mRNA transcript of very low abundance has been described in the rat (Kakar *et al* 1993b), no convincing evidence for the existence of such a transcript was found in the sheep.

The CgA mRNA species displayed similar decreases in abundance from the luteal to the follicular phase. Again this agrees with findings in the rat where CgA levels decrease as the animal ages (Anouar and Duval 1991). Ovariectomy, in the rat, induces an increase in CgA protein and mRNA which can be consequently reversed by oestradiol administration (Anouar *et al* 1991a, b), whilst male rats possess higher pituitary CgA mRNA abundance, possibly due to the relative lack of oestradiol (Fischer-Colbrie *et al* 1992). The lack of an increase in mRNA levels post-surge may be explained by the fact that a further six days remained between the B+96h group and the day 10 luteal phase controls for the required increase to take place. Presumably, this increase would be due to the inability of the low levels of oestradiol in the luteal phase (Campbell *et al* 1990) to suppress CgA mRNA. *In vitro* studies on pituitary adenomas indicate that CgA mRNA levels are not increased by GnRH (Song *et al* 1990). Dexamethasone selectively increases CgA protein and mRNA in rat gonadotrophs (Fischer-Colbrie *et al* 1988). Although this finding suggests a possible role of the adrenal gland in mediating the

post-surge increase in mRNA level, a 4h pretreatment with cortisol significantly reduced GnRH-stimulated LH release from perifused sheep pituitary cells (Nangalama and Moberg 1991). The increasing pulsatile release of LH from the pituitary in the 96h after the LH surge, described in the present study, is not compatible with the expected negative effect due to any adrenal influence. Thus the mechanism by which CgA mRNA up-regulates in the gonadotroph is undefined during the ovine oestrous cycle at present.

CgB mRNA abundance decreased from luteal to follicular phase with no change in abundance over the four days following the LH surge. Although this is suggestive that CgB gene expression is under the negative control of oestradiol, no increases in abundance were observed after immunoneutralisation of oestradiol. However, oestradiol did lead to a non significant decrease in abundance. The regulatory role of oestradiol in other species is not proven. CgB mRNA levels are similar in male and female rats (Fischer Colbrie *et al* 1992). In fact, treatment of human pituitary tumour cells lines *in vitro* with oestradiol increased CgB protein and mRNA levels whereas dexamethasone resulted in a reduction of the mRNA level (Scammell *et al* 1990; Laverriere *et al* 1991). A further study found no effect of dexamethasone on CgB mRNA in gonadotrophs (Fischer-Colbrie *et al* 1988)

Granule synthesis within the gonadotroph appears to be greatest in the luteal phase, as indicated by the highest levels LHβ and granin mRNAs, where the numbers of non polarised cells are highest. The findings that CgA, CgB and SgII are colocalised within the same granule as LHβ and that the mRNAs are apparently regulated in parallel from luteal through follicular phase, suggest a function for the granins that is related to the formation of the secretory granule.

The differing effects of oestradiol and GnRH reported in the rat literature may be a consequence of:

1. different control mechanisms which are required due to the interspecies variation in localisation of the proteins.
2. culture systems which remove cells from their natural environment thus causing the depletion of any potential paracrine regulators of cell function including pulsatile GnRH input.

Furthermore, the apparently anomalous decrease in SgII mRNA after immunoneutralisation of oestradiol, may be an indication of the existence of another suppressive regulator *in vivo*, or an interaction with the LHβ mRNA which was down

regulated with the same treatment. The possibility that oestradiol does act directly to regulate SgII is suggested by the fact that following ovariectomy, it was only possible to reduce SgII mRNA with high concentrations of oestradiol, lower concentrations having no effect (Anouar *et al* 1991a).

Assigning physiological functions to the granin family will be simplified by the identification and localisation of cellular receptors. Currently, no receptors have been characterised. Post translational processing of the granins in other tissues leads to the production of a host of cleavage products with (potential) bioactivity (**1.5.3.**). It is notable that none of the cleavage products so far characterised show any apparent bioactivity in the adenohypophysis. At present, the results of this and other experiments suggest that the possible function of the granins in the gonadotroph is confined to a structural contribution to granule transport and exocytosis, in the case of CgA , CgB and SgII. SgII may also function as a paracrine regulator of an, as yet, undefined cell function due to the nuclear localisation signals contained within its amino acid sequence. The physiological role for Sn in the pituitary is unclear. Ongoing studies have estimated that only around 25% of SgII is processed to Sn in the adenohypophysis (R. Fischer-Colbrie personal communication).

CHAPTER 8

General Discussion

The studies outlined in this thesis have investigated the post-translational control of LH secretion in the Welsh mountain ewe. The results discussed in the previous chapters may be combined to construct a gonadotroph life cycle (Fig. 89). In mid-luteal phase, 80% of gonadotrophs are filled with LHβ immunoreactive granules distributed throughout the entire cytoplasm, thus displaying a non polarised morphology. The high abundance of LHβ mRNA present during luteal phase suggest that the non-polarised gonadotroph is the predominant synthesising form. The 20% of gonadotrophs in which secretory granules are polarised towards the side of the cell nearest the vascular system, may respond to the GnRH input producing LH pulses at this stage of the oestrous cycle. Stereological measurements suggest that smaller granule size classes only are released at this time. Basal LH secretion may result from the spontaneous fusion of small numbers of granules with the plasma membrane. This may occur from both polarised and non-polarised cells. The observation of LHβ immunoreactivity outwith any electron-dense granule structure 24h after an induced LH surge, and the basal systemic LH concentrations measured at that time, suggests that LH not stored in electron dense granules may be secreted in a non pulsatile manner during the early luteal phase. As the follicular phase progresses, declining LHβ mRNA abundance suggests a decrease in hormone synthesis. Once synthesis is complete, cells translocate their LH stores to an intracellular location that favours release. Increasing concentrations of follicular oestradiol (Baird and McNeilly 1981) and the increasing GnRH pulse frequency (Clarke *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994) acting in tandem could mediate the recruitment of gonadotrophs into the polarised state. Studies reported in this thesis indicate that approximately 20% of gonadotrophs are polarised from day 12 luteal phase (Chapter 4) until 36h into the follicular phase (Chapter 6). Around this time, the advent of behavioural oestrus is associated with an increase in the polarised cell cohort to approximately 40% of gonadotrophs. Immediately prior to the LH surge, this increased to 70%. At the onset of the LH surge, the disassembly of the cell cortex may allow the closer association of the secretory granules with the plasma membrane, thus favouring exocytosis. This remodelling of the peripheral cytoskeleton may be under the control of calcium via the protein scinderin which is known to carry out this function in chromaffin cells (Trifaro 1992, 1993). The increasing GnRH pulse frequency which occurs concomitantly with a decreased GnRH

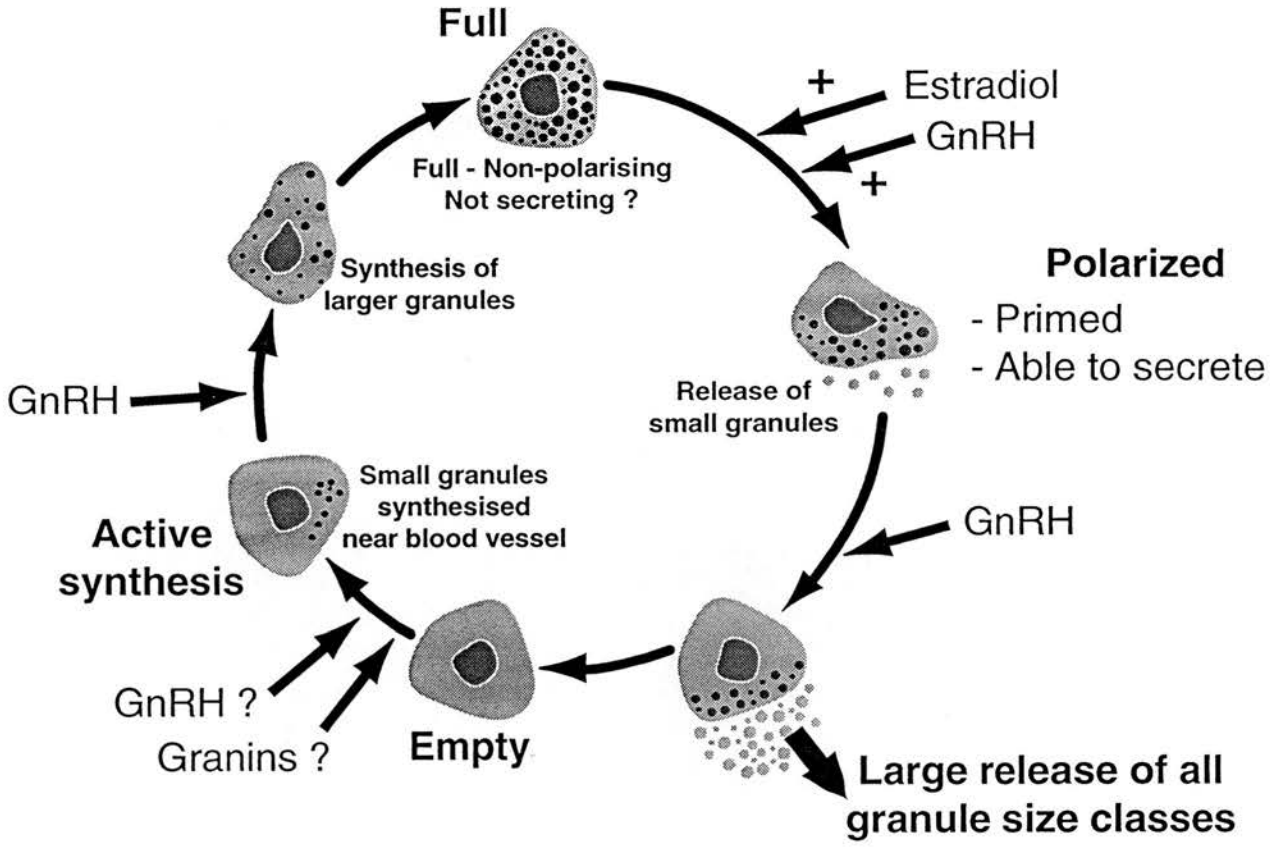


Figure 89. Proposed life cycle of an LH secreting gonadotroph.

pulse amplitude and increased basal secretion of GnRH immediately prior to the preovulatory LH surge (Evans *et al* 1994) may be significant in the control of the scinderin activity. GnRH has been shown to induce a biphasic calcium flux in pituitary cells. Following the GnRH-induced rise in free intracellular calcium, levels return to basal values due to the sequestering of calcium to intracellular stores and its removal via membrane channels (Anderson *et al* 1992). It may be that a submaximal calcium response induced by the decreased amplitude, high frequency GnRH pulses allows a second stimulation before the calcium levels have returned to basal values. Thus, whilst the maximal level of calcium mobilisation within a cell remains unaltered, the overall basal levels may rise. This rise in basal calcium may be critical to the complete disassembly of filamentous actin and hence successful exocytosis. During the preovulatory LH surge, 90% of gonadotrophs are polarised as the population undergoes its period of maximal exocytosis. The increased GnRH receptor mRNA and binding capacity measured at this stage of the cycle (Brooks *et al* 1993) may be a consequence of an increase in the ability of the polarised gonadotroph to respond to the releasing signal from the hypothalamus.

The observations on the numbers of polarised gonadotrophs during the LH surge were made on a group of ewes which had failed to exhibit behavioural oestrus and subsequently been induced to surge with the acute administration of the GnRH agonist buserelin. It is thought that these observations reflect events in the naturally occurring LH surge because:

1. the magnitude of the induced surge was comparable to the natural surge measured in the same experiment (Chapter 4, Fig. 29)
2. measurement of plasma oestradiol concentrations indicated that the animals which had failed to show behavioural oestrus had been exposed to oestradiol concentrations similar to other animal groups in the late follicular phase (Chapter 4, Fig. 31).

Increased plasma oestradiol levels (Hauger *et al* 1977; Pant *et al* 1977; Baird 1978; Baird and McNeilly 1981) and a GnRH surge (Clarke *et al* 1987; Moenter *et al* 1991; Evans *et al* 1994), lead to the generation of the preovulatory LH surge. This is the only stage during the cycle at which gonadotrophs appear to empty their LH stores completely. The complete breakdown of the cell cortex by scinderin, suggested above, may facilitate extrusion of the entire granule population whilst a less extensive disassembly may allow only the smaller granule size classes to be exocytosed. Disassembly of cortical actin by scinderin may only occur above a certain threshold level of calcium. In this case, the calcium transients which return to basal values as a

consequence of the low frequency GnRH pulses during luteal and early follicular phase, will only allow partial actin remodelling. This may restrict exocytosis to the smallest granule classes. Thus the largest secretory granule size classes appear to contribute only to the LH surge and not to the LH pulses present at other times during the cycle.

Following the preovulatory LH surge, LH pulses are absent for around the first 24-48h. LHβ immunoreactivity located in the agranular cytoplasm suggests that the basal levels of LH released and the pituitary LH content measured at this time may be in an agranular form. The production of LH pulses from around 48h after the LH surge onwards coincides with the reappearance of LHβ immunopositive granules. The granules first appear in the aspect of the cell nearest the vascular system. This gives all cells a 'polarised' appearance. The LH pulses present at this time may originate from all cells, some of which later become unresponsive to GnRH and fully regranulate to form the non-polarised cell pool. Alternatively, a small proportion of the total population of gonadotrophs may retain responsiveness after the LH surge and secrete hormone in a pulsatile manner, whilst the remainder, although apparently polarised, refill their LH stores by synthesising granules from one aspect of the cytoplasm. The smallest granule size classes are synthesised first, granule diameter increasing as the animal progresses through luteal phase. The increases in pituitary LH content and secretory granule number occurring post LH surge are not accompanied by a significant change in LHβ mRNA abundance. This suggests that in the early luteal phase, post transcriptional mechanisms may operate to control gonadotrophin translation.

It is now possible to propose an addition to the model for the effects of oestradiol on LH secretion. During the luteal phase when LHβ mRNA is abundant the low oestradiol concentration present may inhibit LH release by preventing gonadotroph polarisation directly. A direct action on GnRH release and hence granule polarisation seems unlikely since the catecholaminergic systems that modulate GnRH release in response to oestradiol are thought to operate predominantly in anoestrus (Meyer and Goodman 1985). Also, the suppression of LH pulse amplitude by oestradiol (Goodman and Karsch 1980; Rawlings *et al* 1984), when applied to the oestrous cycle, may be an indication of low numbers of polarised gonadotrophs and hence of cells contributing to each LH pulse. It is not known at present what effects, if any, high luteal phase progesterone concentrations have on the polarised status of the gonadotroph. Any such effect seems unlikely as progesterone does not suppress pituitary responsiveness to GnRH (Cumming *et al* 1972) but reduces GnRH pulse frequency at the level of the hypothalamus (Goodman and Karsch 1980).

During the follicular phase, oestradiol increases LH pulse frequency (Karsch *et al* 1983). This may be a direct consequence of the recruitment of gonadotrophs into the polarised cohort. It remains to be determined if the oestradiol induced increase in GnRH receptor number (Clarke and Cummins 1984; Brooks *et al* 1993) is restricted to the polarised subpopulation. The studies outlined in this thesis clearly indicate that the preovulatory LH surge is not related to the increased synthesis of LH but to the movement of intracellular stores to a site adjacent to the nearest blood vessel. This suggests that the positive effects of oestradiol, in the sheep, are directed towards increasing GnRH pulse frequency, GnRH receptor number, GnRH receptor mRNA, the polarisation of LH granules within the cytoplasm and not *de novo* synthesis of LH itself.

The failure to establish a long term perifusion system (Chapter 3) may have been related to a failure in the ability of dispersed gonadotrophs to polarise their granule stores. If, as seems likely, the intercellular connective tissue and relationship of the vascular system to the cell play a role in the directional nature of granule translocation, the removal of the gonadotroph from its environment may disrupt the polarised nature of LH secretion. The observation of prolactin granules located in the aspect of the cytoplasm furthest from the point of cell-cell contact, suggests that surrounding cells may assist in the directing of granules away from adjoining cells and hence towards the vascular system. It was clearly noted that collagenase dispersed cells appeared in clumps or chains of cells. The preservation of the correct association of different cell types may be critical to achieve co-ordinated release of LH. Thus, correct cell-cell association together with relatively high cell viability, may have combined to produce pulsatile LH release over a three day period (Chapter 3, Fig. 15). In subsequent dispersions, cell death due to excess collagenase activity together with incorrect cell cell interaction may have contributed to the lack of responsiveness obtained. It may be that the use of dispersed pituitary cell aggregates, as described in the rat by Van Der Schueren *et al* (1982), will allow the maintenance of intercell relationships. The perifusion of pituitary slices may also be more successful although long term culture may be prevented by necrosis. The absence of communication between cells dispersed using trypsin, may explain the loss of the GnRH-stimulated LH response after 24h in perifused cell culture (J. Brooks unpublished observations).

The role of the granin family of proteins in the process of packaging and secretion of LH remains unclear. Throughout the follicular phase CgA, CgB and SgII mRNAs are regulated in parallel with LHβ mRNA abundance, each demonstrating a decrease from

luteal phase through to the preovulatory LH surge. Immunostaining for the three granins investigated was only observed within secretory granules. Cytoplasmic LHβ subunit staining in the agranular gonadotroph cell profiles observed 24h after the onset of the LH surge (Chapter 5) was not accompanied by granin immunostaining. These observations suggest that the granins may be involved in the maintenance of granule structure. The preferential localisation of CgA and CgB in the periphery of the granule indicate a possible role in the exocytotic process. The ability of CgA to undergo increased association with membranes in the presence of calcium (Gorr *et al* 1988; Leiser and Sherwood 1989) and form dimeric and tetrameric complexes (Yoo and Lewis 1992, 1993) suggests an involvement in both the translocation of the granule body during the polarisation process and the fusion of the granule and plasma membrane lipid bilayers during exocytosis. The previous reports of CgB and its plasma membrane association (Chanat and Huttner; unpublished, cited in Chanat *et al* 1993), although not confirmed here, most probably due to the absence of exocytosis at the time of fixation, suggest a function related to the actual fusion pore. This may be either in the maintenance of pore structure or the retrieval of the granule membrane which is apparently returned to the Golgi apparatus following exocytosis (Sandvig *et al* 1992; Sollner *et al* 1993). The localisation of SgII throughout the granule matrix and its lack of association with the membranes is suggestive of a different function. The increased concentrations of SgII which are apparently present within the larger granule size classes (Chapter 7) may indicate a structural role within the granule matrix.

As no SgII or Sn immunoreactivity was observed over the nucleus, no evidence exists to support a paracrine regulatory role due to the putative nuclear localisation signals in the peptide sequence as suggested by Kakar *et al* (1993b). In general, the lack of granin immunoreactivity outwith the secretory granule profiles argues against their role as a paracrine regulator. However, it may be that post translational cleavage products, unrecognised by the antibodies used in the current studies, may have regulatory properties. The characterisation of receptors for the granins will assist in the definition of an endocrine function.

It has been suggested the granins may function as a type of chaperone protein, assisting in the packaging of hormones (Rosa *et al* 1985). This potential function has recently been discredited due to relative lack of the granins in relation to the co stored hormones (Winkler and Fischer Colbrie 1992). This putative function cannot be dismissed solely due to apparently unfavourable stoichiometry. It may be that low levels of the proteins are sufficient to enable granule condensation to occur. Indeed, the exclusion of other

proteins when the granins are experimentally induced to aggregate (Gerdes *et al* 1989) may be an artifact of supraphysiological granin concentrations.

The regulation of the granin mRNA species by physiologically significant hormones provides circumstantial evidence of some role in the reproductive process. It is of particular interest that all three granin mRNAs are negatively regulated from luteal through the follicular phase. Presumably, the rising follicular oestradiol concentration at this time effects this decrease in preference to the stimulatory effects of GnRH previously reported. GnRH has been shown to increase both CgB (human pituitary adenoma) (Song *et al* 1990) and SgII (female rat) mRNAs have been shown to increase in this way (Kakar *et al* 1993b).

The finding that progressively larger numbers of gonadotrophs appear to be recruited into the polarised state towards the onset of the LH surge allows a new interpretation of the events surrounding LH secretion. The decrease in LHβ, CgA, CgB and SgII mRNAs as the numbers of polarised cells increases throughout the follicular phase suggests that transcription of LH, CgA, CgB and SgII protein may occur predominantly in non-polarised cells. Furthermore, the induction of the polarised state may either lead to, or be a consequence of the up regulation of GnRH receptor numbers and the increased level of GnRH receptor mRNA observed in late follicular phase (Brooks *et al* 1993). Ultrastructural *in situ* hybridisation together with immunolocalisation, particularly of the GnRH receptor will clarify the translation and expression states of the different gonadotroph morphologies.

The absence of the three granin proteins from the light dense bodies (LDBs) present in the natural oestrous cycle and induced by the administration of oestradiol suggests that these organelles may not be secretory granules. In the natural oestrous cycle, these structures occur at an extremely low incidence. They may be part of the trans Golgi network (TGN) from which LH has not yet budded off to form a granule. The extremely high level of LHβ immunoreactivity present suggests that the presence of the granins may determine the ability of the TGN to form secretory granules. If this hypothesis were true, then a lack of the granins in the lumen of the TGN would lead to an accumulation of LH which would be unable to bud off the membrane thus forming a LDB.

It is possible that the pharmacological dose of oestradiol, which led to increased numbers of LDBs, caused a decrease in the granin mRNAs and hence a lack of the

intact protein within the TGN lumen. Throughout the ovine follicular phase, the decline in LHβ mRNA may be under the negative regulatory influence of oestradiol. The postulated mechanism of LDB formation depends on one the following:

1. the decline in granin mRNAs and hence translated product is more acute than occurs for LHβ subunit.
2. the rates of decline are the same but the effect is more marked on the granins due to their lower concentration within the cell.

As a putative oestrogen response element (ERE) has been localised at around 1.7kb upstream of the transcription start site of the ovine LHβ gene (Dr. P. Brown; personal communication), this indicates that LHβ may be regulated directly by a genomic effect of oestradiol. The hypothesis that LHβ subunit production is influenced less quickly than the granins would therefore seem untenable. Although this favours the second proposed mechanism of action, the fact that a pharmacological dose of oestradiol was used may indicate that an unphysiological response was produced. Supraphysiological doses of oestradiol may be able to increase LHβ transcription. Increased activity of rat LHβ promoter activity in response to oestradiol administration has been shown following the transfection of a construct composed of LHβ containing the putative ERE and the herpes simplex virus thymidine kinase promoter fused to the chloramphenicol acetyltransferase gene, into the pituitary GH_3 cell line (Shupnik *et al* 1989b). It is therefore possible for the LHβ gene to respond in two ways to oestradiol, although negative regulation appears to be the predominant form *in vivo* in the gonadotroph. An increase in LHβ subunit without adequate levels of the granins may lead to an inability to bud from the TGN, thus generating the large numbers of light dense bodies observed. It is not thought that LDBs are immature secretory granules because of their lack of abundance at all stages of the oestrous cycle, including the luteal phase when synthetic activity is apparently at its height. Furthermore, there are no mechanisms currently defined which could explain the insertion of the granin proteins into the secretory granule after it had left the TGN.

Although the data produced so far in this thesis and elsewhere indicates a central role for the granins in the production of LH granules, further experiments are required to confirm this. In particular, the use of a gonadotrophin-secreting cell line would allow the manipulation of the granin content of the cells. Transfection of each of the granin genes could be used to systematically introduce each of the granin proteins to the intracellular environment. The ability of the cell to form granules and effect their exocytosis could then be assessed. For such a system to produce results which replicated the physiological situation, gonadotrophin secretion would have to involve the

polarisation of secretory granules to one aspect of the cell cytoplasm. This may involve the duplication of the correct orientation of basement membranes and connective tissue types which surround the gonadotroph *in vivo*. Immunostaining of tissue sections to determine the composition of the intercellular environment is required. This may assist in the setting up of a successful culture system, perhaps in conjunction with the cell aggregates or pituitary slices described earlier.

It is possible that a greater understanding of the mechanisms that underlie the exocytosis of the gonadotrophins will facilitate improved clinical strategies with regard to the management of fertility in the future. The ability to disrupt a specific mechanism relating to LH granule exocytosis may provide a means by which long term contraceptive therapies can easily be achieved. Immunisation against a particular protein involved in the LH secretory pathway would have fertility management applications both in human and veterinary medicine.

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